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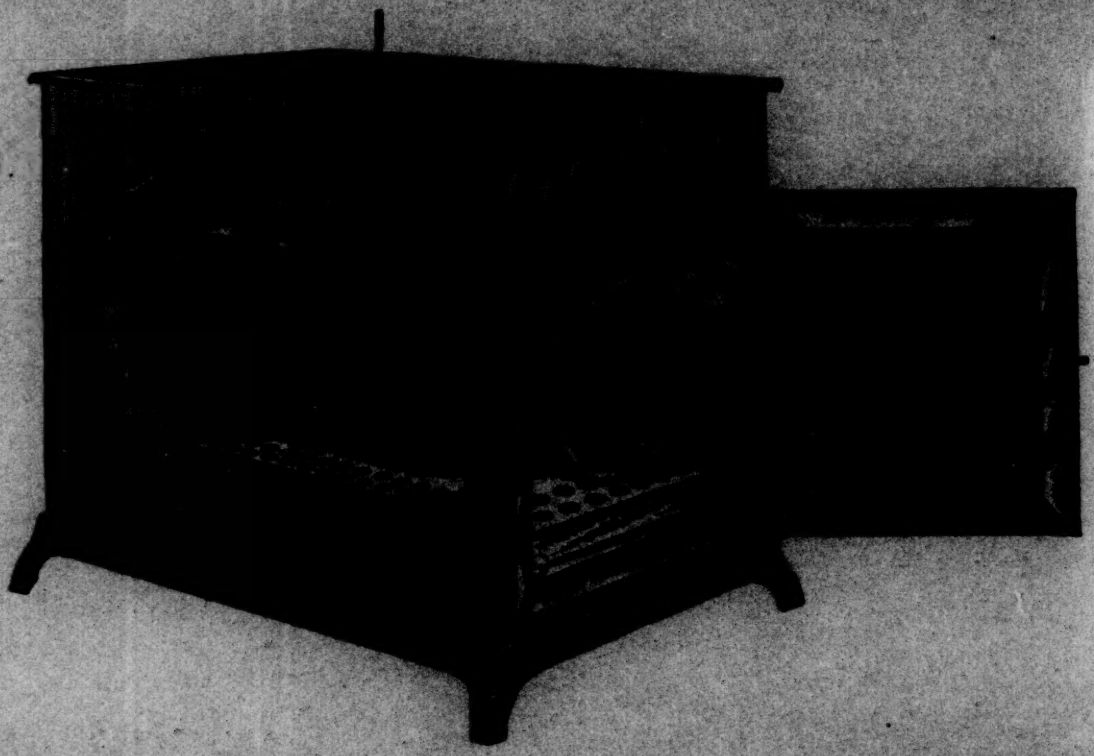

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THE CASEIN OF GOAT'S MILK.

By ALFRED W. BOSWORTH and LUCIUS L. VAN SLYKE.

(From the Chemical Laboratory of the New York State Agricultural Experiment Station, Geneva.)

(Received for publication, January 8, 1916.)

In studying the composition of goat's milk in this laboratory, we found it necessary to know the percentage of phosphorus in the casein prepared from such milk and also the power of this casein to combine with bases to form caseinates. We have prepared casein from goat's milk by the method used in our study of the casein of cow's milk.[1] However, we found it even more difficult to remove all of the calcium phosphate, owing to the fact, shown in the following article, that goat's milk contains both tri- and di-calcium phosphate; but, by repeating the process of precipitation and redissolving in the presence of neutral potassium oxalate, we finally obtained a preparation in which the percentage of ash was about 0.36.

Below is given the analysis of a preparation of casein made from goat's milk together with the analysis of casein of cow's milk.

Percentage of Constituents in Moisture-Free and Ash-Free Casein.

	From goat's milk.	From cow's milk.
Ash	0.36	0.06
Carbon	52.50	53.50
Hydrogen	7.16	7.13
Nitrogen	15.67	15.80
Phosphorus	0.71	0.71
Sulfur	0.71	0.72
Oxygen (by difference)	23.25	22.08

[1] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1913, xiv, 203.

Relation of Casein of Goat's Milk to Bases.

By applying the details of the methods already published by us[2] we find that the casein of goat's milk combines with bases to form the same series of salts (caseinates) as in the case of the casein of cow's milk. The results of our work are briefly embodied in the following summary.

(1) 1 gm. of casein combines with 9×10^{-4} gm. equivalents of base to form a compound (caseinate) neutral to phenolphthalein. (2) 1 gm. of casein combines with 1.125×10^{-4} gm. equivalents of ammonia, sodium, or potassium to form a soluble caseinate; there is no other combination of casein with a base containing less than 1.125×10^{-4} gm. equivalents of base for 1 gm. of casein. (3) 1 gm. of casein combines with 2.25×10^{-4} gm. equivalents of calcium to form a caseinate soluble in water. This caseinate is insoluble in water in the presence of more than a trace of any soluble salt of calcium, barium, or strontium. (4) 1 gm. of casein combines with 1.125×10^{-4} gm. equivalents of calcium to form a caseinate insoluble in water. This compound is soluble in a warm 5 per cent solution of sodium chloride, the solvent action being due to an interchange of bases by double decomposition, with the consequent production of soluble sodium caseinate.[3]

Valency and Molecular Weight of Casein of Goat's Milk.

From the preceding data, it can be seen that the valency of casein from goat's milk, like that of casein from cow's milk, is 8.

$$\frac{9 \times 10^{-4}}{1.125 \times 10^{-4}} = 8$$

The molecular weight would, therefore, be $\frac{1}{1.125 \times 10^{-4}} = 8{,}888$,

and the equivalent weight would be $\frac{8{,}888}{8} = 1{,}111$.

On the basis of the sulfur content we would have:

$$\text{Molecular weight} = \text{N} \left(\frac{32.07}{0.71} \right) \times 100 = \text{N}\ (4{,}393).$$

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[2] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1913, xiv, 211.

[3] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1913, xiv, 217.

If N equals 2, the molecular weight equals 8,786.

On the basis of the amount of phosphorus contained in casein, we have:

$$\text{Molecular weight} = N\left(\frac{31.04}{0.71}\right) \times 100 = N\ (4{,}372).$$

If N equals 2, the molecular weight equals 8,744.

SUMMARY.

On the basis of the analytical results obtained in the study of the compounds formed by casein of goat's milk with bases, taken together with the amount of phosphorus and sulfur found in such casein, the molecular weight is 8,888, and the valency of the protein molecule in basic caseinates is 8.

THE SOLUBLE AND INSOLUBLE COMPOUNDS OF GOAT'S MILK.

By ALFRED W. BOSWORTH and LUCIUS L. VAN SLYKE.

(From the Chemical Laboratory of the New York State Agricultural Experiment Station, Geneva.)

(Received for publication, January 8, 1916.)

When milk is brought into contact with a porous earthenware filter, the serum, consisting of the water and the compounds in true solution, passes through the filter, while the compounds insoluble in water, which are present in suspension (fat and calcium phosphate), or in the form of colloids (casein), remain on the surface of the filter. The details of the method used in separating milk-serum from the other constituents of milk have been fully given elsewhere.[1]

By determining the amounts of the constituents present in the whole milk and in the serum, we are enabled to determine by difference the quantities present in the milk in suspension or colloidal solution.

Table I gives the figures obtained by us in working with four samples of goat's milk, in which only the more prominent constituents were determined.

A study of the data contained in Table I enables us to show the general relation of the constituents of milk to the constituents of milk-serum. The following form of statement furnishes a clear summary of the facts.

1. Milk constituents in true solution in milk.	2. Milk constituents partly in solution and partly in suspension or colloidal solution.	3. Milk constituents entirely in suspension or colloidal solution.
Sugar.	Albumin.	Fat.
Potassium.	Inorganic phosphates.	Casein.
Sodium.	Calcium.	
Chlorine.	Magnesium.	
	Citrates.	

[1] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1915, xx, 136.

TABLE I.

Constituents of Goat's Milk.

Constituents.	Sample 1.			Sample 2.			Sample 3.			Sample 4.		
	In original milk, per 100 cc.	In milk-serum, per 100 cc.	Milk constituents in serum.	In original milk, per 100 cc.	In milk-serum, per 100 cc.	Milk constituents in serum.	In original milk, per 100 cc.	In milk-serum, per 100 cc.	Milk constituents in serum.	In original milk, per 100 cc.	In milk-serum, per 100 cc.	Milk constituents in serum.
	gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*
Fat	3.80	0.00	0.00	2.20	0.00	0.00	3.70	0.00	0.00	3.00	0.00	0.00
Sugar	5.22	5.22	100.00	4.82	4.82	100.00	4.52	4.52	100.00	4.45	4.45	100.00
Nitrogen, total	0.520	0.114	21.92	0.492	0.104	21.13	0.450	0.078	17.33	0.404	0.075	18.56
" in casein	0.340	0.000	00.00	0.353	0.000	00.00	0.316	0.000	0.00	0.267	0.000	00.00
" " albumin	0.090	0.035	38.89	0.059	0.024	40.67	0.060	0.012	20.00	0.067	0.010	14.92
" " other proteins	0.090	0.079	87.78	0.080	0.080	100.00	0.074	0.066	89.19	0.070	0.065	92.86
Citric acid	0.1307	0.0991	75.82	0.1485	0.1322	89.02	0.1712	0.1283	74.89	0.1531	0.1379	90.07
Phosphorus, total	0.1206	0.0502	41.63	0.0906	0.0390	43.05	0.0862	0.0392	54.48	0.0841	0.0413	49.11
" organic	0.0153	0.000	00.00	0.0159	0.000	00.00	0.0142	0.0000	00.00	0.0120	0.000	00.00
" inorganic	0.1053	0.0502	47.67	0.0747	0.0390	52.21	0.0720	0.0392	54.44	0.0721	0.0413	57.28
Calcium	0.1632	0.0414	25.37	0.1500	0.0534	35.60	0.1240	0.0351	28.31	0.1144	0.0374	32.69
Magnesium	0.0204	0.0128	62.75	0.0212	0.0132	62.26	0.0206	0.0128	62.19	0.0236	0.0144	61.02
Potassium	0.1700	0.1724	100.00	0.1656	0.1784	100.00	0.2284	0.2284	100.00	0.2084	0.2084	100.00
Sodium	0.0380	0.0380	100.00	0.0400	0.0396	100.00	0.0352	0.0352	100.00	0.0372	0.0376	100.00
Chlorine	0.1578	0.1581	100.00	0.1811	0.1839	100.00	0.2171	0.2168	100.00	0.2597	0.2665	100.00

Properties and Composition of Portion of Milk in Suspension or Colloidal Solution.

Some of the constituents of milk are suspended in the form of solid particles in such an extremely fine state of division that they pass through the pores of filter-paper, and do not settle as a sediment on standing, but remain permanently afloat. They cannot be seen except by ultramicroscopic methods. When substances are in such a condition, they are said to form a colloidal solution. In passing milk through the Pasteur-Chamberland filtering-tube, the constituents in suspension as solid particles, and in colloidal solution, are retained in a solid mass on the outside of the tube and can therefore be readily obtained for study.

Appearance.—When prepared by the method of filtration previously described, the insoluble portion of fat-free milk collecting on the outside of the filtering-tube is grayish to greenish

white in color, of a glistening appearance, and of gelatinous consistency. When dried without purification by treatment with alcohol, etc., it resembles dried white of egg.

Behavior with Water.—The deposit of insoluble milk constituents on the outside of the filtering-tube, when removed and shaken vigorously in a flask with distilled water, goes into suspension, and the mixture has the opaque, white appearance of the original milk. The deposit is, of course, more or less mixed with adhering soluble constituents, but can be readily purified by shaking with distilled water and filtering several times. The purified material goes readily into suspension on shaking with water, and, if treated with a preservative, will remain indefinitely without change other than the separation of fat-globules. It has been held by some that the citrates of milk perform the function of holding the insoluble phosphates in suspension, but this belief is not supported by the behavior of the insoluble portion, as shown in our experiments.

Reaction.—A suspension of the insoluble constituents of milk, prepared in the manner described above, is alkaline to phenolphthalein. We purified the deposit obtained from 1,000 cc. of milk and made a suspension of it in water; it was found to be neutral to phenolphthalein, but, after the addition of 10 cc. of neutral potassium oxalate, it developed a strongly alkaline reaction to phenolphthalein. We interpret this behavior to mean that the insoluble portion of goat's milk contains an alkaline phosphate, which, being insoluble, does not affect the indicator; but the addition of a solution of neutral potassium oxalate decomposes the insoluble phosphate, with the production of soluble tri-potassium phosphate, which reacts alkaline to phenolphthalein, as will be shown later (page 181).

Composition.—As shown in Table I, the insoluble portion of goat's milk contains as chief constituents, fat, protein, inorganic phosphates, calcium, and magnesium. The fat can be separated from the other constituents by extraction with ether or by removal from the original milk by means of centrifugal force. We have previously shown that albumin may be adsorbed to some extent by casein[2] and thus partly retained on the filter. In view

[2] Van Slyke and Bosworth, *J. Biol. Chem.*, 1915, xx, 138.

of these conditions, our studies have been limited to the acids (casein and phosphates) and the bases (calcium and magnesium) present in this portion of the milk.

Relation between Acids and Bases in the Insoluble Portion of Goat's Milk.—In order to bring out clearly the relation existing between the bases and the acids in the insoluble portion of the milk, we have prepared Tables II and III. Table II is obtained from the figures in Table I, and Table III is obtained by converting the percentage figures of Table II into gm. equivalents per

TABLE II.

Amounts of Casein, Inorganic Phosphorus, Citric Acid, Calcium, and Magnesium in the Insoluble Portion of Goat's Milk.

Sample No.	Casein.	Inorganic phosphorus.	Citric acid.	Calcium.	Magnesium.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
1	2.17	0.0551	0.0316	0.1218	0.0076
2	2.25	0.0357	0.0163	0.0966	0.0080
3	2.01	0.0328	0.0429	0.0889	0.0078
4	1.70	0.0308	0.0152	0.0770	0.0092

TABLE III.

Amounts of Acids and Bases in Table II Expressed as Gm. Equivalents Per 100 Cc. of Milk.

Sample No.	Casein as octavalent acid.	Phosphorus as di-basic acid.	Citric acid as tri-basic acid.	Sum of values of acids.	Calcium.	Magnesium.	Sum of values of bases.	Excess of base.
1	19.5×10^{-4}	35.5×10^{-4}	4.9×10^{-4}	59.9×10^{-4}	60.9×10^{-4}	6.3×10^{-4}	67.2×10^{-4}	7.3×10^{-4}
2	20.3×10^{-4}	23.0×10^{-4}	2.5×10^{-4}	45.8×10^{-4}	48.3×10^{-4}	6.7×10^{-4}	55.0×10^{-4}	9.2×10^{-4}
3	18.1×10^{-4}	21.2×10^{-4}	6.7×10^{-4}	46.0×10^{-4}	44.5×10^{-4}	6.5×10^{-4}	51.0×10^{-4}	5.0×10^{-4}
4	15.3×10^{-4}	19.9×10^{-4}	2.4×10^{-4}	37.6×10^{-4}	37.2×10^{-4}	7.7×10^{-4}	44.9×10^{-4}	7.3×10^{-4}

100 cc. of milk. In making these calculations, we have considered the casein of goat's milk to be octavalent, 1 gm. combining with 9×10^{-4} gm. equivalents of base to form a compound neutral to phenolphthalein (page 174). The figures for organic phosphorus are obtained by subtracting the amount of phosphorus in the casein (0.71 per cent) from the total phosphorus.

The last column of Table III shows that the amounts of bases in the insoluble portion of goat's milk are in excess of the amounts required to form with the acids that are present salts neutral to

phenolphthalein. There is reason to believe that this excess of base is present in combination with the phosphorus in the form of tri-calcium phosphate. The evidence supporting this belief is furnished by an examination of the insoluble portion of goat's milk obtained by separation through centrifugal force in a cream-separator.[3]

The bowl of a cream-separator was filled with about 1,000 cc. of fat-free milk and was whirled for 2 hours at a speed of 5,000 revolutions a minute. The liquid portion of the milk was then

TABLE IV.

Composition of Insoluble Portion or "Separator Slime" of Goat's Milk.

Deposit formed by whirling two 2 hour periods.	Casein.	Total phosphorus.	Phosphorus in casein.	Phosphorus as phosphate.	Calcium.	Ratio of organic to insoluble inorganic phosphorus.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	
1st 2 hrs.	75.56	2.57	0.54	2.03	4.46	1:3.72
2nd 2 hrs.	73.17	2.59	0.53	2.06	4.40	1:3.81

TABLE V.

Amounts of Acids and Bases in Table IV Expressed as Gm. Equivalents per 100 Cc. of Milk.

	Casein as gm. equivalents of acid.	Phosphate as gm. equivalents of di-basic acid.	Sum of gm. equivalents of casein and phosphates.	Gm. equivalents of calcium.	Excess of calcium as gm. equivalents.
In 1st deposit	68×10^{-3}	132×10^{-3}	200×10^{-3}	226×10^{-3}	26×10^{-3}
" 2nd "	65.9×10^{-3}	132×10^{-3}	198.7×10^{-3}	220×10^{-3}	21.3×10^{-3}

taken from the bowl and the deposit ("separator slime"), which had collected on the walls of the separator-bowl, was removed, treated with alcohol and ether, and dried in the manner described.[4] This liquid portion of the milk that had been whirled for 2 hours was again placed in the separator-bowl and whirled for an additional 2 hours, when the deposit was again removed and treated as before. After the removal of the second deposit, the liquid portion of the milk remaining in the separator was nearly as

[3] Van Slyke and Bosworth, *J. Biol. Chem.*, 1915, xx, 144.

[4] Van Slyke and Bosworth, *J. Biol. Chem.*, 1915, xx, 145.

clear as whey, since most of the suspended phosphates and casein had been deposited on the walls of the bowl during the whirling.

The results of the analysis of the dried deposit obtained after each 2 hour period of whirling are given in Table IV.

Expressing the data in Table IV in the form of gm. equivalents per 100 cc. of milk, we obtain the figures contained in Table V.

These figures show an amount of calcium in excess of that required to form salts neutral to phenolphthalein with the acids present, casein and phosphoric acid. It should be stated here that these deposits of "separator slime" contain no citric acid. When the deposit is suspended in water in the presence of phenolphthalein, no color appears; but, if 1 cc. of a neutral solution of potassium oxalate is added, a pink color appears, becoming more intense after standing for 1 or 2 minutes. The most obvious explanation of this behavior is that the addition of neutral potassium oxalate results in a reaction between it and the tri-calcium phosphate, tri-potassium phosphate being formed and turning the indicator pink.

The Acidity of Goat's Milk and Its Serum.

When the determination of the acidity of goat's milk and its serum is made by adding neutral potassium oxalate and filtering previous to titration, according to the method recently published by us,[5] results are obtained as given in Table VI.

There is observable a marked difference between the acidity of the whole milk and that of its serum. This condition is accounted for by the following explanation. In normal goat's milk, which is nearly neutral, there is present in suspension di-calcium phosphate; this phosphate, in the presence of the water in the milk, undergoes more or less decomposition, for, according to the work of Cameron and Hurst,[6] water in contact with a solid phase containing calcium phosphate dissolves more acid than base from the solid phase. The result of this action is that the insoluble portion of the milk becomes more basic than the whole milk, as shown above, while the soluble portion becomes more

[5] Van Slyke and Bosworth, *J. Biol. Chem.*, 1914, xix, 73.

[6] Cameron, F. K., and Hurst, L. A., *J. Am. Chem. Soc.*, 1904, xxvi, 905.

TABLE VI.

Acidity of Goat's Milk and Its Serum.

Sample No.	0.1 N alkali required to neutralize 100 cc. of	
	Milk.	Serum.
	cc.	cc.
1	1.4	6.6
2	3.0	5.6
3	0.6	4.8
4	0.3	5.8
5	0.0	5.0
6	0.0	3.0

acid. When the solid and liquid portions of milk are separated by treatment on a porous porcelain filter, the soluble acid passes into the serum, the basic compound remaining in the insoluble portion.

In determining the acidity of milk, it should be remembered that the presence of di-calcium phosphate furnishes conditions favorable to the formation of acid phosphates and tri-calcium phosphate, as already shown,[5] resulting in an increased requirement of alkali for neutralization and apparently a higher acidity if the milk is titrated directly. For this reason the lower acidity obtained after the removal of calcium by neutral potassium oxalate is believed to represent the real acidity of the milk. This point is well illustrated by the following figures.

TABLE VII.

Sample No.	0.1 N alkali required to neutralize 100 cc. of milk.	
	Before addition of neutral potassium oxalate.	After addition of neutral potassium oxalate.
	cc.	cc.
1	17.6	1.5
2	21.0	3.0
3	26.0	3.2
4	25.6	8.0
5	20.6	1.4

Compounds in Goat's Milk.

Based on the result of our study of the composition of goat's milk, the following arrangement is tentatively suggested as representing the forms in which the constituents may be present:

		per cent	per cent
Fat			3.80
Proteins, combined with calcium			3.10
Milk-sugar			4.50
Salts			
Di-calcium phosphate		0.092	
Tri-calcium "		0.062	
Di-magnesium "		0.068	
Tri-magnesium "		0.024	
Mono-potassium "		0.073	0.94
Potassium citrate		0.250	
Potassium chloride		0.160	
Calcium "		0.115	
Sodium "		0.095	
Total solids			12.34

SUMMARY.

1. Goat's milk contains two general classes of compounds, those in true solution and those in suspension or colloidal solution. These two portions can be separated for study by filtering milk through a porous earthenware filter like the Pasteur-Chamberland filtering-tube.

2. Serum prepared from fresh milk of goats is nearly transparent, with a faint greenish yellow tinge and slight opalescence. The following constituents are in true solution: sugar, potassium, sodium, chlorine. The following are partly in solution and partly in suspension or colloidal solution: albumin, inorganic phosphates, calcium, magnesium, citrates. The following are entirely in suspension or colloidal solution:- fat, casein.

3. The insoluble portion of milk, freshly prepared and moist, is grayish to greenish white in color, of a glistening appearance, and of gelatinous consistency. When shaken with water, it goes into suspension, forming a mixture having the opaque, white appearance of milk. Such a suspension is neutral to phenolphthalein. When purified, the insoluble portion consists of neu-

tral calcium caseinate (casein Ca_4), di- and tri-calcium, and magnesium phosphates.

4. The insoluble portion, suspended in water and treated with neutral potassium oxalate, reacts alkaline to phenolphthalein, indicating the presence of tri-calcium phosphate. This is shown also by a quantitative comparison of the amount of bases and acids.

5. The acidity of the serum is considerably greater than that shown by the milk after treatment with neutral calcium oxalate. This is accounted for by the presence of di-calcium phosphate.

6. The data presented furnish a basis for suggesting the forms of compounds in which the salts may be present.

A COMPARISON OF THE COMPOSITION OF COW'S MILK, GOAT'S MILK, AND HUMAN MILK.

By ALFRED W. BOSWORTH and LUCIUS L. VAN SLYKE.

(*From the Chemical Laboratory of the New York State Agricultural Experiment Station, Geneva.*)

(Received for publication, January 8, 1916.)

We have published statements attempting to indicate the individual forms or compounds in which the salts exist in cow's milk and in human milk.[1] The same has been done for goat's milk in the preceding paper. It is a matter of interest to bring these results together in order to see in what respects the three kinds of milk differ. It is understood, of course, that the arrange-

Compounds in Cow's Milk, Goat's Milk, and Human Milk.

Compounds.	Cow's milk.	Goat's milk.	Human milk.
	per cent	*per cent*	*per cent*
Fat	3.90	3.80	3.30
Milk-sugar	4.90	4.50	6.50
Proteins, combined with calcium	3.20	3.10	1.50
Salts	0.901	0.939	0.313
Di-calcium phosphate	0.175	0.092	0.000
Tri-calcium "	0.000	0.062	0.000
Mono-magnesium phosphate	0.103	0.000	0.027
Di-magnesium "	0.000	0.068	0.000
Tri-magnesium "	0.000	0.024	0.000
Mono-potassium "	0.000	0.073	0.069
Di-potassium "	0.230	0.000	0.000
Potassium citrate	0.052	0.250	0.103
Sodium "	0.222	0.000	0.055
Potassium chloride	0.000	0.160	0.000
Sodium "	0.000	0.095	0.000
Calcium "	0.119	0.115	0.059

[1] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1915, xx, 151. Bosworth, A. W., *ibid.*, 1915, xx, 707.

ment of the constituents is based upon a limited amount of work. We know that there is variation in the quantitative relations of the different compounds. There remains much work to be done in applying our methods to the study of milks produced under a variety of known conditions. The present results are, therefore, tentative in character and are likely to be modified by more extensive work.

In the preceding table the figures which have a special interest are those relating to the salts, and we notice the following points in relation to these compounds.

Phosphates.

Cow's Milk.—The insoluble phosphate is di-calcium phosphate; tri-calcium, di- and tri-magnesium phosphates do not appear to be present. The soluble phosphates are mono-magnesium and di-potassium, which constitute about two-thirds of the total phosphates.

Goat's Milk.—This differs from cow's milk (1) in containing tri-calcium, di- and tri-magnesium, and mono-potassium phosphates, and (2) in containing no mono-magnesium or di-potassium phosphates.

Human Milk.—This differs noticeably from both cow's milk and goat's milk in containing no insoluble phosphates, only the soluble compounds, mono-magnesium and mono-potassium phosphates. The phosphates in human milk are much less in amount than in cow's or goat's milk.

Citrates.

All three milks contain potassium citrate, while cow's milk and human milk contain sodium citrate also.

Chlorides.

Chlorides are present in goat's milk in much larger amounts than in cow's milk or human milk; the amount in cow's milk is considerably larger than in human milk. In cow's milk and human milk the chloride appears to be calcium chloride, while in goat's milk potassium and sodium chlorides are also present.

Total Salts.

The total amount of salts in human milk is about one-third that in cow's milk or goat's milk. The number of different salts appears to be greatest in goat's milk and least in human milk.

CHEMICAL CHANGES IN THE SOURING OF MILK.

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The investigation described in this paper was undertaken for the purpose of learning what chemical changes take place in the insoluble and soluble constituents of milk when souring under ordinary conditions. This work has been carried out with the use of the method previously employed by us in studying the condition of casein and salts in milk.[1] Briefly stated, the method separates milk into a soluble portion and an insoluble portion, making use of a porous porcelain filter as the means of separation; the chemical composition of each portion is determined.

Our study of the chemical changes taking place in the constituents of milk as the result of souring originated several years ago in connection with a study of problems relating to the manufacture and ripening of cheese, especially the American cheddar cheese, which is the type that has been most largely made in this country. The first work was carried out also in connection with a study of cottage, or sour-milk cheese.[2] In the manufacture of most kinds of cheese, the early stages are largely associated with the formation of lactic acid, the amount of acid being governed especially by the character or type of the cheese made. We thus have in the case of different kinds of cheese the common process of lactic acid fermentation; the chemical changes resulting are essentially the same in kind, whether the amount of acid is small or large, but are, of course, more extensive in proportion as the amount of acid increases. As might readily be supposed, the control of the lactic acid fermentation plays a very important rôle in the manufacturing processes of all types of cheese.

[1] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1915, xx, 136.
[2] Van Slyke, L. L., and Hart, E. B., *Am. Chem. J.*, 1904, xxxii, 145.

We have carried out numerous experiments in studying the changes that take place in milk as a result of souring and the results have been uniform in agreement. We shall, therefore, instead of presenting all of our data, give such as will fully illustrate the ascertained facts. We will give first the analytical results obtained with milk at the end of 60 hours and then a set of results obtained at frequent intervals during a period of 96 hours.

Changes in Milk in 60 Hours Caused by Souring.

Before considering the detailed changes that are produced in the constituents of milk during the various stages of progressive souring, we will show the final changes that take place when the souring process has been practically completed, which under ordinary conditions should be in about 60 hours.

4 liters of fresh milk, divided into two portions, of 2 liters each, were treated as follows: To one portion chloroform was added at once and the mixture was passed through a porous porcelain filter. The second portion was allowed to sour at room temperature after inoculation with a culture containing *Bacterium lactis acidi* and *Bacterium lactis aerogenes*. At the end of 60 hours, this was filtered through a porous porcelain filter. Analysis was made of the fresh milk, of the serum of the fresh milk, and of the serum of the sour milk, the results of which are given in Table I.

A study of the results in Table I shows in general that the main effects produced by the souring of milk are the conversion of more or less milk-sugar into lactic acid, which causes precipitation of casein and makes soluble the other insoluble constituents. Stated in more detail, we notice the following results:

1. The amount of sugar in milk is decreased through the action of the lactic acid bacteria. In this case there is a loss of 1.27 gm. for 5.75 gm. of sugar per 100 cc. of milk, or 22 per cent. The amount of lactic acid formed is 1.124 gm., or 88.5 per cent of the sugar decomposed is converted into lactic acid.

2. The citric acid of milk completely disappears, being decomposed into acetic acid and carbon dioxide by the action of *Bacterium lactis aerogenes*.

3. Those inorganic constituents of normal milk that are insoluble are made soluble by the acid resulting from bacterial action.

4. The albumin, part of which in normal milk fails to pass through a porous porcelain filter, is so changed in sour milk as to pass completely through such a filter.

5. Calcium caseinate of normal milk is completely converted into the free protein, uncombined with any base, which is precipitated, and calcium lactate is formed, soluble in the serum.

TABLE I.

Changes in the Condition of Milk Constituents as a Result of Souring.

Constituents.	Original milk, 100 cc.	Serum from fresh milk.		Serum from sour milk.	
		Serum, 100 cc.	Percentage of milk constituents in serum.	Serum, 100 cc.	Percentage of milk constituents in serum.
	gm.	*gm.*		*gm.*	
Sugar	5.75	5.75	100.00	4.48	—
Casein	3.07	0.00	0.00	0.00	0.00
Albumin	0.506	0.188	37.15	0.506	100.00
Nitrogen in other compounds	0.049	0.049	100.00	0.049	100.00
Citric acid	0.237	0.237	100.00	0.000	0.00*
Phosphorus, inorganic	0.087	0.056	64.40	0.090	100.00
Calcium	0.144	0.048	33.33	0.148	100.00
Magnesium	0.013	0.007	53.85	0.014	100.00
Potassium	0.120	0.124	100.00	0.120	100.00
Sodium	0.055	0.057	100.00	0.058	100.00
Chlorine	0.076	0.081	100.00	0.079	100.00
Ash	0.725	0.400	55.17	0.690	95.17**
Lactic acid	0.000	0.000	0.00	1.124	—†

* During the process of souring the citric acid is completely changed by the action of *Bacterium lactis aerogenes* into acetic acid and carbon dioxide, as shown by Bosworth, A. W., and Prucha, M. J., *J. Biol. Chem.*, 1910, viii, 479.

** In determining the total ash of milk, the organic phosphorus of the casein is included in the ash as phosphoric acid, but in the serum this organic phosphorus is not present and therefore the amount of ash in serum from sour milk is less than that in fresh milk.

† Some of the lactic acid is adsorbed by the casein and therefore not all of it is found in the serum (Van Slyke, L. L., and Van Slyke, D. D., *Am. Chem. J.*, 1907, xxxviii, 383).

Changes in Milk at Different Stages of Souring.

In addition to the ultimate changes, it is desirable to know something of the rate of chemical change taking place when milk sours. We have carried on numerous sets of experiments, but present here the results of only one, which is typical of all under the same conditions.

The milk used in this experiment was fresh morning milk, which had been pasteurized and run through a separator to remove the fat. The milk was inoculated with a pure culture of *Bacterium lactis acidi* and kept at a temperature of about 32.2°C. (90°F.), samples being taken for analysis at intervals and chloroformed at once in order to prevent further bacterial action. Sampling began after the milk had stood 4 hours and continued at intervals of an hour or less for 16 hours; after 24 hours samples were taken every 24 hours up to the end of 96 hours.

The constituents to which attention was given are the following: (1) sugar, (2) lactic acid, (3) acidity before and after passing through porcelain filter, (4) nitrogen in serum, (5) nitrogen as albumin in serum, (6) soluble and insoluble inorganic phosphorus, (7) soluble and insoluble calcium and magnesium, (8) calcium as di-calcium phosphate, (9) calcium derived from calcium caseinate.

The methods by which the amounts of total, soluble, and insoluble calcium, magnesium, and phosphorus were obtained have been fully described in our previous work.[1]

Considerable time was devoted to attempts to determine separately the amounts of free lactic acid and combined lactic acid, but no satisfactory method could be devised for making such distinction in the presence of acid phosphates. This is due to the conditions existing in sour milk, where we have present acid calcium phosphate and calcium lactate, and, in advanced stages of souring, some free lactic acid. Extraction of a solution containing lactates in the presence of acid phosphates results in the following reaction, which gives an apparent quantity of free lactic acid much greater than the actual amount:

$$CaH_4P_2O_8 + Ca(C_3H_5O_3)_2 = 2CaHPO_4 + 2HC_3H_5O_3$$

In a solution containing free lactic acid, lactates, and phosphates, the lactate and phosphate undergo mutual reaction, forming free

lactic acid and di-calcium phosphate. While we find it possible to extract with ether all the free lactic acid from a solution containing these compounds, we are unable to tell with accuracy at what point the conversion of lactate into free lactic acid begins. It is possible by extraction to remove the lactic acid of the lactate completely from a solution containing calcium lactate and acid calcium phosphate provided the phosphate is present in excess, but we have thus far found no satisfactory way of making a separate determination of free lactic acid in the presence of these salts by extraction. We hope, however, to do this later by determination of the free hydrogen ions.

TABLE II.

Composition of Fresh Milk before Souring.

	In 100 cc.		In 100 cc.
	gm.		*gm.*
Nitrogen, total	0.5964	Phosphorus, inorganic, insoluble	0.0399
" as casein	0.4704	Calcium, total	0.1571
" " albumin	0.0762	" soluble	0.0427
" other than casein and albumin	0.0498	" insoluble	0.1144
Nitrogen in serum	0.0770	" " as $CaHPO_4$	0.0515
Phosphorus, total	0.1039	" combined with protein	0.0629
" in casein	0.0211	Magnesium, total	0.0158
" inorganic	0.0828	" soluble	0.0112
" " soluble	0.0429	" insoluble	0.0046

Before giving the results of the work for the sour milk in its various progressive stages of change, we give in Table II a statement of the compounds present in the fresh milk previous to souring, as a convenience for reference in interpreting the data in Table III.

Conversion of Milk-Sugar into Lactic Acid in the Souring of Milk.

Table III gives (1) the amount of sugar in the milk when fresh and at intervals up to the end of 96 hours, and (2) the amount of lactic acid found. The lactic acid was determined by extraction with ether and conversion into zinc lactate.

The fermentation of the milk-sugar, under the conditions of the experiment, is slow during the initial period but between the 10th and 25th hours most of the change takes place. With increase of lactic acid beyond 0.7 per cent, bacterial activity is much reduced. Between the 48th and 96th hours only a slight change in sugar takes place. Attention is called to the apparent discrepancy at 48 and 96 hours in the relation of the amount of lactic acid formed to the amount of sugar decomposed. Apparently more acid is formed than can be accounted for by the amount of sugar fermented. This is due to the fact that the acetic acid produced by the fermentation of the citric acid is extracted by ether and weighed with the lactic acid.

TABLE III.

Formation of Lactic Acid in the Souring of Milk.

Age of milk when sampled.	100 cc. of milk.			Milk-sugar changed.	Fermented sugar changed into lactic acid.
	Sugar.	Amount of sugar changed.	Lactic acid.		
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
Fresh.	5.30	—	0.000	—	—
10	5.07	0.23	0.200	4.3	87
12	4.83	0.47	0.330	8.9	70
14	4.68	0.62	0.513	11.7	82
19	4.58	0.72	0.671	13.6	93
25	4.42	0.88	0.665	16.6	75.5
48	4.30	1.00	1.052	18.9	—
96	4.26	1.04	1.124	20.0	—

In the last column of Table III we give the proportion of fermented milk-sugar that was changed into lactic acid. The proportions vary from 70 to 93 per cent. It is known that products other than lactic acid are formed, varying in kind and amount according to the conditions of the fermentation process.

Effect of the Souring of Milk upon Acidity of Milk and Milk-Serum.

Table IV gives (1) the amount of total acidity in the fresh milk and that found at intervals during the process of fermentation; (2) the amount of acids in the serum; (3) the amount of acids

in the portion of the milk that does not pass through the porous porcelain filter; and (4) the amount of lactic acid.

The acidity of the insoluble portion of the milk is obtained by difference. The amount or degree of acidity, in the case of the milk and milk-serum, is expressed in terms of 0.1 N alkali required to produce neutrality to phenolphthalein after removal of cal-

TABLE IV.

Acidity of Milk, Milk-Serum, etc.

Age of milk when sampled.	Acidity expressed as cc. 0.1 N alkali required to neutralize 100 cc. of milk.				Lactic acid.
	In milk, total.	In serum.	In insoluble portion.	Increase of acidity in milk.	
hrs.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Fresh.	10.0	7.8	2.2	—	—
4	12.0	8.2	3.8	2.0	1.7
5	14.0	9.6	4.4	4.0	3.8
6	15.6	10.0	5.6	5.6	5.3
7	19.6	11.8	7.8	9.6	9.2
8	21.0	13.6	7.4	11.0	10.9
9	26.0	15.6	10.4	16.0	15.7
10	33.0	17.0	16.0	23.0	22.0
11	37.0	20.6	16.4	27.0	26.7
11½	42.4	24.0	18.4	32.4	31.3
12	47.6	27.0	20.6	37.6	36.6
13	55.6*	30.2	25.4*	45.6*	45.6
13½	62.0	30.2	31.8	52.0	52.0
14	67.1	32.0	35.1	57.1	57.1
25	84.0	41.6	42.4	74.0	74.0
48	127.0	53.6	73.4	117.0	117.0
72	133.0	59.0	74.0	123.0	123.0
96	135.0	62.4	72.6	125.0	125.0

* As explained in the text above, the figures expressing total acidity in milk from 13 to 96 hours inclusive are obtained by calculation, owing to the curdling of the milk.

cium by neutral potassium oxalate. The amount of lactic acid was determined by extraction and conversion into zinc lactate.

After 12 hours the milk curdled and the acidity could not be satisfactorily determined by titration. The figures given in Table IV, Column 2, for total acidity in milk after 12 hours are obtained by adding 10 (the amount of acid in the fresh milk) to the amount

of lactic acid found by direct determination. The figures in Column 4 under acidity of "insoluble portion" of milk and in Column 5 under "increase of acidity in milk" after 12 hours are based also upon the calculated amount of total acidity.

Attention is called to the following points connected with the results given in Table IV:

1. Increase of Total Acidity in Milk.—There is an increase of acidity in the milk; this is more or less rapid for the intervals of the first 24 hours; the increase during the second 24 hours is much less than during the first, and after 48 hours further increase is insignificant. The acidity of fresh milk is due to acid phosphates. The increase is due to the production of lactic acid resulting from the decomposition of milk-sugar. This is clearly shown by a comparison of the last two columns, in which the figures representing the increase of acidity in milk run closely parallel with those representing the amount of lactic acid obtained by direct determination.

2. Acidity of Serum.—The acidity of the serum increases during the whole observed period of 96 hours, but the increase is relatively small after the first 24 hours. The increase of acidity in the milk-serum is due to the increased formation of lactic acid in the milk. We should expect, owing to the solubility of the compounds producing acidity, to find the increase of acidity in the serum closely equal to the amount of increase of lactic acid. However, a comparison of the figures shows that the amount of acid in the serum does not increase as rapidly as lactic acid. The explanation that most readily suggests itself for this condition is that there is adsorption of the acid compounds by the particles of precipitated casein, as already stated in the third foot-note in connection with Table I.

3. Acidity of Insoluble Portion.—During the process of souring, the calcium is removed from the calcium caseinate of milk, and the resulting form of casein is able to neutralize alkali. The decalcified casein constitutes the portion of the insoluble part of milk that possesses acid properties, but, owing to its property of adsorbing acids, the insoluble part of souring milk, prepared by the method of filtration through a porous porcelain filter, shows a higher acidity than that due to the base-combining power of the decalcified casein.

Effect of the Souring of Milk upon Insoluble Calcium and Inorganic Phosphorus.

The insoluble calcium in fresh milk is in combination (1) with phosphoric acid as $CaHPO_4$ and (2) with casein as Ca_4 caseinate. With the formation of lactic acid, the di-calcium phosphate is converted into mono-calcium phosphate ($CaH_4P_2O_8$), and the calcium caseinate is changed, first, into caseinates containing less

TABLE V.

Proportions of Insoluble Calcium and Inorganic Phosphorus Dissolved in the Souring of Milk.

Age of milk when sampled.	Insoluble calcium in 100 cc. of milk.			Percentage of insoluble calcium in milk dissolved in case of		Insoluble inorganic phosphorus in 100 cc. of milk.	Percentage of insoluble inorganic phosphorus dissolved.
	Total.	In combination as $CaHPO_4$.	In combination as caseinate.	$CaHPO_4$	Caseinate.		
hrs.	*gm.*	*gm.*	*gm.*			*gm.*	
Fresh.	0.1144	0.0515	0.0629	—	—	0.0399	—
4	0.1102	0.0470	0.0632	8.7	0.0	0.0364	8.7
5	0.1090	0.0457	0.0633	11.2	0.0	0.0354	11.2
6	0.1041	0.0431	0.0610	16.3	3.2	0.0334	16.3
7	0.0974	0.0418	0.0556	18.8	11.7	0.0324	18.8
8	0.0922	0.0374	0.0548	27.4	13.0	0.0290	27.4
9	0.0775	0.0319	0.0456	38.0	27.6	0.0247	38.0
10	0.0648	0.0255	0.0393	50.5	37.6	0.0198	50.5
11	0.0560	0.0180	0.0380	65.0	39.7	0.0140	65.0
12	0.0368	0.0049	0.0319	90.5	—	0.0038	90.5
13	0.0191	0.0019	0.0172	96.3	72.7	0.0015	96.3
$13\frac{1}{2}$	0.0154	0.0000	0.0154	100.0	75.5	0.0000	100.0
15	0.0092	—	0.0092	—	85.4	—	—
19	0.0088	—	0.0088	—	86.0	—	—
25	0.0000	—	0.0000	—	100.0	—	—

calcium and, finally, into uncombined casein. Table V gives for different intervals of the process of souring the following determinations: (1) insoluble calcium, total; (2) calcium in combination as $CaHPO_4$; (3) calcium in calcium caseinate; (4) amount of calcium as $CaHPO_4$ that is dissolved; (5) amount of calcium in calcium caseinate that is dissolved; (6) insoluble inorganic phosphorus; and (7) inorganic insoluble phosphorus dissolved. The values given for fresh milk are obtained from Table II.

In studying the data in Table V, we call attention to the following summary of the results:

1. Insoluble calcium and phosphorus in combination as $CaHPO_4$ begin to go into solution as $CaH_4P_2O_8$ and calcium lactate in a few hours and the action continues with increasing rapidity most of the time until the solution is complete at the end of $13\frac{1}{2}$ hours under the conditions of the experiments.

2. The insoluble calcium in combination with casein does not appear to be acted upon by the lactic acid as quickly as does that in $CaHPO_4$. The calcium in calcium caseinate is not completely converted into calcium lactate until the fermentation has been going on about 24 hours. Broadly speaking, the calcium in $CaHPO_4$ is acted upon about twice as rapidly as that in calcium caseinate during the first 12 hours under the conditions of our experiments.

It may be added here that determinations of insoluble magnesium were also made. The results show that the insoluble magnesium goes into solution completely in $11\frac{1}{2}$ hours, which is somewhat sooner than the insoluble calcium in $CaHPO_4$.

Effect of the Souring of Milk upon Milk-Albumin.

In Table II the per cent of nitrogen in the milk used in our experiments is given as 0.0762 gm. per 100 cc. of milk; the per cent of nitrogen in forms other than casein and albumin is 0.0498 gm., and the per cent of nitrogen in serum is 0.770 gm. per 100 cc. of milk. The amount of nitrogen in the serum includes that in forms other than casein and albumin, and the amount of this nitrogen subtracted from the nitrogen found as albumin in milk gives the amount of albumin in the serum, which is 0.0272 gm. per 100 cc. of milk. The amounts of total nitrogen and of albumin nitrogen in serum have been determined at intervals during the souring of milk and the results are given in Table VI.

The significant point that attracts attention in connection with the data in Table VI is the increase of nitrogen in the serum. This increase is due to albumin since there was no appreciable solution of casein under the conditions of our work. The formation of lactic acid is apparently responsible for this change. The change may be due to decrease of adsorption of albumin by

casein consequent upon the change produced in casein from caseinate to free casein, or it may be due to a combination of base with albumin in milk and a gradual separation of base and protein in much the same manner as happens with casein.

TABLE VI.

Nitrogen in Serum and Its Relation to Albumin.

Age of milk when sampled.	Total nitrogen in serum from 100 cc. of milk.	Percentage of increase of nitrogen in serum.	Nitrogen as albumin in serum from 100 cc. of milk.	Percentage of albumin of milk in serum.
hrs.	*gm.*		*gm.*	
Fresh.	0.0770	—	0.0272	35.7
10	0.0950	23.4	0.0452	60.0
11	0.0970	26.0	0.0472	62.0
12	0.1120	45.5	0.0622	81.6
13	0.1180	53.2	0.0682	90.0
14	0.1270	65.0	0.0772	100.0

SUMMARY.

1. Fresh milk was analyzed for its soluble and insoluble constituents, a porous porcelain filter being used to make separation. Another portion of the same milk was inoculated with a culture containing *Bacterium lactis acidi* and *Bacterium lactis aerogenes*. At the end of 60 hours determinations were made of the soluble and insoluble portions. (*a*) About 22 per cent of the milk-sugar is changed by the lactic acid bacteria, 88.5 per cent of the amount changed being converted into lactic acid. (*b*) Citric acid completely disappears. (*c*) The insoluble inorganic constituents of the fresh milk are made soluble by the lactic acid. (*d*) Albumin of sour milk passes through the porcelain filter completely. (*e*) Calcium caseinate is changed into free protein and precipitated, the calcium forming lactate.

2. To study the rate and extent of chemical change under given conditions, fresh, pasteurized, separated skim-milk was inoculated with a pure culture of *Bacterium lactis acidi* and kept at 32.2°C. Samples were taken for analysis at intervals during 96 hours. (*a*) Most of the change in milk-sugar occurs between the 10th and 24th hours. When the milk contains 0.7 per cent of lactic acid, the bacterial activity is much reduced. (*b*) The acidity

increases most rapidly during the first 24 hours, the rate of increase diminishing after that. The increased acidity of the serum is due to increase of lactic acid. In the insoluble portion of the milk the free casein is the acid constituent. (*c*) Calcium combined as $CaHPO_4$ goes into solution completely in $13\frac{1}{2}$ hours. Calcium combined as caseinate is acted upon more slowly, complete solution requiring about 24 hours. (*d*) The amount of albumin nitrogen in serum increases with increase of acidity; all the albumin of the milk appears in the serum in 14 hours.

THE RATE OF UREA EXCRETION.

FIRST PAPER.

A CRITICISM OF AMBARD AND WEILL'S LAWS OF UREA EXCRETION.

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Ambard and Weill[1] have reached the conclusion that the rate of excretion of urea by the normal kidney is dependent on only two factors, the concentration of urea in the blood and in the urine. Their method was to collect urine over as short a period of time as was consonant with accuracy in collection, and to compare the rate of urea excretion with the concentration of urea present in the urine and in a specimen of blood removed at about the middle of the period of urine collection. When the concentration of urea in the urine in one individual happened to be the same as that found in another, they noted that the rate of urea excretion varied proportionally to the square of the concentration of urea in the blood. Further, when the blood urea concentration was the same in different individuals, the rate of urea excretion varied inversely as the square root of the urine concentration. These two observations were expressed in mathematical form and combined in a single formula, a correction for body weight being introduced. They showed that the resultant of this formula in all normal people under the most widely different conditions of urea and water intake was a constant. A departure from the constant was taken as indicating an abnormality in kidney function. The formula has been widely used by clinicians—mainly in France—as a means whereby minor deviations from the normal may be detected.

McLean and Selling,[2] and McLean[3] have recently published a large number of observations on the concentration of urea in the blood and urine of normal individuals. The resultant of Ambard's formula in these cases was, they considered, an approximate constant, and they recommend the method for clinical use.

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[1] Ambard, L., and Weill, A., *J. physiol. et path. gén.*, 1912, xiv, 753.
[2] McLean, F. C., and Selling, L., *J. Biol. Chem.*, 1914, xix, 31.
[3] McLean, F. C., *J. Exp. Med.*, 1915, xxii, 212.

The two separate laws on which Ambard's formula is based have not yet been confirmed. McLean and Selling, and McLean deal only with the combined formula, and it is noteworthy that the observations on which Ambard and Weill established these empirical laws are very few in number. The main proof that they are correct is allowed to rest on the approximate constancy of the combined formula. The reason for this lies in the fact that in any series of individuals it is only a chance when either the urine concentration or the blood concentration happen to be the same. There is no way to make them the same. Therefore even in a considerable number of observations, there may be relatively few in which this constancy is attained.

It so happened that in the course of an investigation into the factors regulating the rate of urea excretion, which has been carried on for over a year, we had accumulated figures which bear directly on the question of the validity of Ambard and Weill's laws. After the appearance of McLean and Selling's apparent confirmation of Ambard and Weill's theories, we thought it well to review these data. We found that we had a large number of instances in which either the urine or the blood urea concentration of different individuals was the same or within the limits of error of the methods we employed. These cases have been grouped together in the tables. According to the laws of urea excretion there should be a different constant for each group, to which each instance within the group conforms, when the formulas given by Ambard and Weill are applied. We found that there was no approach to anything that could be even roughly described as a constant. Variations of over 100 per cent were frequent. It is evident from the tables that other factors than the concentration of urea in the blood and urine must commonly intervene in the process of urea excretion.[4]

We believe it is important to demonstrate that the factors governing the excretion of urea by the kidneys are not fully known, for the impression that they are not only known but are measurable with mathematical accuracy is likely to retard further investigation. The approximate constancy of the combined formula, which after all is only roughly approximate, is due in part to the

[4] These tables may seem unnecessarily long, but we expect to refer to them in subsequent communications.

tendency for increased urea concentration in the blood to be accompanied by an increased rate of urea excretion. But in large part it is to be ascribed to its mathematical construction. The more variable factors—the concentration in the urine, the volume of urine, and the amount of urea in the urine—occur as the square or fourth roots of their values. Their disturbing effect on the constancy of the resultant of the formula is thus greatly reduced, while the only factor used without such modification—the concentration in the blood—is itself the most constant quantity used.

Methods.

The urease method of urea estimation was used. With the blood a method almost identical with that of Van Slyke and Cullen was employed, except that during the last few months we have mixed the blood with finely ground soy bean meal, instead of using a purified extract. We never aerated for less than 40 minutes. Most of the estimations recorded here were done in duplicate. In the urine Marshall's original method was used with two modifications. The urine was collected in vessels containing 5 cc. of N H_2SO_4 for every hour's collection, and before adding the urease the contents of the flask were made just alkaline to rosolic acid. The reasons for these changes will be detailed in a paper on urea excretion, which will be published soon. All specimens were diluted to the same volume. The activity of the soy bean extract was determined every day by estimations of known urea solutions. Double determinations were carried out for every specimen, one without and one with the addition of a known amount of urea. The subjects were instructors and students between the ages of 20 and 35, all free from any evidence of disease of the kidneys. The majority underwent an independent investigation of their power to excrete large amounts of urea in which a marked uniformity of functional power was established.

The Relationship between the Concentration of Urea in the Blood and the Rate of Urea Excretion, when the Concentration of Urea in the Urine Is the Same.

The error in the estimation of the concentration of urea in the urine depends on the combined errors in the measurement of the volume of urine and of the amount of urea.

In measuring the volume fractions of 1 cc. were not recorded, so that a volume recorded as 50 cc. might actually be an amount from 49.5 to 50.5 cc. An error of ± 0.5 cc. has therefore been assumed. In regard to the urea estimation we have found, as

Marshall pointed out, that the urease ferment falls a little short of being absolutely quantitative in its action. This, however, is an error in one direction and is relatively constant. The greatest source of error arises from the not very sharp end-points in the titrations. It would seem that an assumed error of ± 1 per cent would certainly be well within the actual limits of error. These combined errors have been adopted as a means of classifying our material. All cases in which the concentration of urine falls within the sum of these limits of error are grouped as having, as far as can be ascertained, the same concentration of urea in the urine. The actually recorded concentration is given for each case. These groups of cases in which the urine concentration is constant have been arranged in order of ascending magnitude of urine concentration, and the cases within the groups in order of descending rates of urea excretion. The rate of urea excretion is expressed as the quantity of urea eliminated in 1 hour, since that was the interval of time during which the collection of urine was made in most instances. When the conditions were such that the volume of urine was large, the time of collection was $\frac{1}{4}$ of an hour, and in some cases when the volume was small, the period of collection was 2 hours. For convenience of reference these cases have been expressed in terms of 1 hour's excretion. In all cases blood was removed from an arm vein at or near the middle of the time of urine collection.

A study of the data thus given in Table I shows that there is no constant relationship between blood urea concentration and the rate of excretion in cases in which the urine concentration is constant.[5]

[5] Ambard and Weill's first law states that when the concentration of urea in the urine is constant, the rate of urea excretion varies proportionally to the square of the concentration of urea in the blood, or

$$\frac{(\text{Blood concentration})^2}{\text{Rate of excretion}} = \text{Constant.}$$

The expression of this law which is employed by them is

$$\frac{\text{Blood concentration}}{\sqrt{\text{Rate of excretion}}} = \text{Constant.}$$

This formula has been used by us. It is perhaps worthy of note that it is to

There are 135 instances in which a comparison of the blood concentration and the rate of excretion may be made between pairs of cases with the same urine concentration; in 92 (68.1 per cent) the rate of excretion was greater with higher blood concentration, and in 43 (31.8 per cent) the rate of excretion was less. It may be said, then, that in cases where the concentration in the urine is the same, there is a tendency for the rate of excretion to be more rapid when the blood concentration is higher, but at most it is one which is frequently obscured. There are many examples where the opposite seems to hold, as for instance the two cases where one with a blood concentration of 0.0232 per cent excretes 0.98 gm. of urea, while the other with a blood concentration of 0.0450 per cent eliminates only 0.39 gm.

The groups in Table I comprise examples of wide variation in rate of urea excretion corresponding to differences in intake of urea or of urea-forming food. It was conceivable that under uniform conditions it might be possible to establish a more evident connection between blood concentration and rate of urea excretion. In Table II only those subjects are included who were under the same conditions as regards nitrogen and water consumption. Here also, however, it will be noted that there are cases where a greater rate of urea excretion is associated with a lower percentage of blood urea.

some extent this particular mathematical form which is responsible for the relatively smaller percentage variation in the constants for the cases within the various groups seen in Table I as compared with those in Table III. The rate of excretion is the most variable of all the factors, and when it is reduced to its square root an appearance of greater uniformity is obtained. In the combined formula a correction for body weight is introduced, on the assumption that a heavy man will have a higher rate of excretion than a light one, other things being equal. We have introduced this factor in the same manner as used by Ambard and Weill, expressing the body weight in relation to the standard weight of 70 kg. Further, in order to make our formula identical with theirs, we have converted our hourly rates of excretion into 24 hour quantities, and our blood concentrations into gm. per liter. The formula then reads

$$\frac{\text{Gm. of urea per liter of blood}}{\sqrt{\text{Hourly rate of excretion} \times 24 \times \frac{70}{\text{Kg. of body weight}}}} = \text{Constant.}$$

The Relationship between the Concentration of Urea in the Urine and the Rate of Urea Excretion when the Concentration of Urea in the Blood Is the Same.

In 90 duplicate estimations of blood urea there was an average variation of 1.5 mg.[6] The greatest variation was 6.6 mg. The results recorded are not, however, the average of two determinations, but the higher of two. The reason for this is that, apart from overtitration, positive errors are unlikely. If any alkali were blown over there would be a large positive error, but we have never seen this occur. It appears to us that the cause of variation in duplicate estimations, apart from the titration error, is due in practically all cases to incomplete decomposition of urea or to incomplete removal of ammonia. Perhaps the physical conditions in the mixture of blood, alkali, and soy bean powder or extract may sometimes be such as either to protect a trace of urea from the action of the ferment, or to hinder a little ammonia from being carried over in the air current, since a variable degree of clumping and agglutination of the material is often observed at the commencement of the aeration and towards its close.

We cannot say what the actual error is, but this is immaterial, since we require to fix a degree of variation which is certainly well within the actual limits of error. We have arbitrarily chosen 0.6 mg. as corresponding to this requirement. All cases in which the difference in blood concentration was not greater than 0.6 mg. have therefore been grouped together. The same arrangement of the groups and of the cases within the groups has been adopted as in Table I.

The figures in Table III show that there is no constant relationship between the concentration of urea in the urine and the rate of urea excretion in cases in which the blood concentration is the same.[7]

[6] In a large series of duplicate estimations carried out since these were completed, the average error has been only 0.5 mg.

[7] Ambard's second law of urea excretion states that when the concentration of urea in the blood is the same, the rate of urea excretion varies inversely as the square root of the concentration of urea in the urine, or.

$$\text{Rate of excretion} \times \sqrt{\text{Urine concentration}} = \text{Constant.}$$

The group constants have been worked out in the same way as for Table I, introducing the correction for body weight and converting the con-

In 136 comparisons between pairs of cases with the same blood concentration there were 91 instances (65.9 per cent) in which the rate of excretion was less with higher urine concentration, and 45 instances (34.0 per cent) in which it was greater with higher urine concentration. Here again all that can be said is that there appears to be a tendency for the rate of urea excretion to be slowed with increased urine concentration in cases in which the blood concentration is the same.

In Table IV subjects with the same blood concentration who were on a diet with the same nitrogen and water content have been grouped. No marked difference from Table III is apparent.

There are a number of instances where both the urine and blood urea concentrations are the same in different individuals or in the same individual at different times. If Ambard and Weill's theories are correct, these cases should show the same rate of urea excretion. They have been grouped in Table V. Wide variations in the rate of urea excretion are seen in these cases. There could not be any clearer indication than this that other factors besides urea concentration are responsible for the rate of urea excretion.

CONCLUSION.

The rate of urea excretion in man varies under physiological conditions in a manner which cannot be explained by the concentrations of urea in the blood and urine.

There is a tendency for an increased rate of urea excretion to exist with higher blood urea concentration in cases in which the urine concentration is the same, and for an increased rate of urea excretion to be accompanied by a lowering of the urea concentration in the urine in cases in which the blood concentration is the same.

This relationship, however, is one which is frequently obscured, even in individuals under the same conditions as regards nitrogen and water intake.

Other factors than urea concentration are important in determining the rate of urea excretion by the normal kidney.

centration of urea in the urine into gm. per liter. The formula therefore reads

$$\text{Hourly rate of excretion} \times 24 \times \frac{70}{\text{Kg. of body weight}} \times \sqrt{\text{Gm. of urea per liter of urine}} = \text{Constant.}$$

TABLE I.

Groups of Cases in Which the Concentration of Urea in the Urine Was the Same.

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Urine concentration per 100 cc.	Blood concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's first law.
		gm.	*gm.*	*cc.*	*gm.*	
Boy	0.91	0.155	0.0324	760	1.18	0.0639
Add	1.05	0.157	0.0402	600	0.94	0.0826
Add	1.05	0.171	0.0414	640	1.096	0.0787
Add	1.05	0.172	0.0354	634	1.094	0.0675
Add	1.05	0.180	0.0300	632	1.14	0.0560
Add	1.05	0.181	0.0312	596	1.08	0.0598
Boy	0.91	0.178	0.0336	584	1.04	0.0706
Ca	0.84	0.197	0.0450	922	1.81	0.0746
Add	1.05	0.196	0.0408	688	1.35	0.0700
J	1.14	0.198	0.0399	590	1.17	0.0701
Mo	0.84	0.198	0.0384	508	1.00	0.0855
Ca	0.84	0.205	0.0384	822	1.81	0.0635
Add	1.05	0.202	0.0489	600	1.21	0.0884
Mo	0.84	0.215	0.0402	508	1.095	0.0855
Mo	0.84	0.219	0.0402	496	1.088	0.0855
V	0.95	0.63	0.0225	151	0.96	0.0482
Add	1.05	0.64	0.0300	143	0.91	0.0636
K	1.03	1.00	0.0216	93	0.93	0.0450
Cr	1.06	1.00	0.0210	75	0.75	0.0482
B	1.11	1.06	0.0240	87	0.93	0.0482
K	1.03	1.04	0.0337	84	0.87	0.0728
V	0.95	1.16	0.0225	64	0.74	0.0550
O	1.09	1.16	0.0204	62	0.72	0.0470
V	0.95	1.17	0.0270	51	0.60	0.0730
G	0.94	1.14	0.0288	34	0.39	0.0970
Ca	0.84	1.25	0.0390	83	1.03	0.0857
Bo	0.81	1.27	0.0261	73	0.93	0.0614
K	1.03	1.28	0.0246	56	0.72	0.0581
We	1.13	1.25	0.0180	42	0.52	0.0482

TABLE I—*Continued.*

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Urine concentration per 100 cc.	Blood concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's first law.
		gm.	*gm.*	*cc.*	*gm.*	
Ca	0.84	1.30	0.0367	81	1.05	0.0718
Add	1.05	1.29	0.0322	80	1.03	0.0631
K	1.03	1.31	0.0216	43	0.57	0.0578
Ch	1.09	1.35	0.0379	121	1.35	0.0638
M	1.08	1.34	0.0156	29	0.39	0.0490
M	1.08	1.43	0.0558	216	3.09	0.0624
Ca	0.84	1.45	0.0292	90	1.30	0.0571
Add	1.05	1.44	0.0322	44	0.63	0.0807
Add	1.05	1.59	0.0585	107	1.71	0.0892
Add	1.05	1.58	0.0472	53	0.84	0.1025
Add	1.05	1.63	0.0540	79	1.29	0.0945
J	1.14	1.62	0.0252	52	0.84	0.0526
Sh	1.00	1.65	0.0330	37	0.61	0.0863
C	0.93	1.71	0.0246	56	0.96	0.0533
K	1.03	1.71	0.0360	47	0.80	0.0812
F	0.90	1.74	0.0240	42	0.73	0.0605
Wi	1.08	1.72	0.0246	34	0.58	0.0634
Add	1.05	1.71	0.0276	28	0.48	0.0795
Sh	1.00	1.73	0.0292	27	0.47	0.0870
Ch	1.09	1.73	0.0252	26	0.45	0.0737
K	1.03	1.78	0.0307	39	0.69	0.0745
Sh	1.00	1.77	0.0337	23	0.41	0.1072
Bb	0.81	1.87	0.0300	47	0.88	0.0724
Add	1.05	1.91	0.0322	45	0.86	0.0692
Wa	1.58	1.90	0.0250	32	0.61	0.0520
Add	1.05	1.97	0.0577	109	2.14	0.0789
Add	1.05	1.99	0.0495	104	2.06	0.0695
Mc	1.19	1.95	0.0246	50	0.97	0.0468
Sh	1.00	1.94	0.0330	24	0.47	0.0984
Add	1.05	1.95	0.0315	20	0.39	0.1002
Add	1.05	2.01	0.0465	60	1.21	0.0841
S	1.08	2.00	0.0240	30	0.60	0.0610

TABLE I—*Continued.*

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Urine concentration per 100 cc.	Blood concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's first law.
		gm.	*gm.*	*cc.*	*gm.*	
Add	1.05	2.06	0.0765	121	2.49	0.0966
W	1.11	2.08	0.0492	118	2.46	0.0607
Add	1.05	2.05	0.0367	51	1.04	0.0717
Ai	1.19	2.07	0.0282	39	0.81	0.0585
Add	1.05	2.06	0.0300	18	0.37	0.0980
B	1.11	2.23	0.0516	84	1.87	0.0733
Mo	0.82	2.22	0.0485	48	1.07	0.1053
Ad	0.99	2.21	0.0438	43	0.95	0.0923
Add	1.05	2.23	0.0427	41	0.91	0.0892
P		2.21	0.0282	30	0.67	
O	1.09	2.34	0.0546	110	2.58	0.0665
Add	1.05	2.35	0.0346	51	1.20	0.0630
Mo	0.82	2.34	0.0232	42	0.98	0.0529
Add	1.05	2.33	0.0450	17	0.39	0.1430
Add	1.05	2.38	0.0495	71	1.70	0.0755
Mo	0.82	2.42	0.0271	44	1.07	0.0590
Add	1.05	2.46	0.0442	39	0.96	0.0899
Br	1.01	2.40	0.0252	20	0.48	0.0740
Ch		2.61	0.0435	68	1.77	
So	0.98	2.64	0.0427	40	1.05	0.0860
Mo	0.82	2.68	0.0435	38	1.02	0.0970
Jo	1.14	2.63	0.0375	37	0.97	0.0726
Add	1.05	2.78	0.0682	78	2.17	0.0925
Add	1.05	2.75	0.0607	69	1.89	0.0881
C	0.93	3.15	0.0624	87	2.74	0.0805
Do	1.00	3.23	0.0651	80	2.59	0.0828
Boy	0.91	3.22	0.0590	72	2.32	0.0830
Cr	1.06	3.21	0.0558	70	2.25	0.0737
K	1.03	3.15	0.0672	68	2.14	0.0925
T	0.86	3.15	0.0252	48	1.51	0.0451
Jo	1.14	3.31	0.0397	25	0.828	0.0835
Jo	1.14	3.29	0.0397	23	0.756	0.0874

TABLE I—*Concluded.*

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Urine concentration per 100 cc.	Blood concentration per 100 cc.	Volume of urine in 1 hr.	Urine excreted in 1 hr.	Constants by Ambard's first law.
		gm.	*gm.*	*cc.*	*gm.*	
E.	1.08	3.55	0.1230	101	3.60	0.1270
Wa.	1.58	3.56	0.0678	69	2.46	0.0704
Ad.	0.90	3.54	0.0750	68	2.41	0.0992
F.	0.90	3.65	0.0558	66	2.41	0.0774
G.	0.94	3.63	0.0612	52	1.89	0.0935
Bo.	1.03	3.72	0.0672	58	2.16	0.0920
Ai.	1.19	3.69	0.0690	58	2.14	0.0885
So.	0.98	3.71	0.0382	20	0.74	0.0917
Jo.	1.14	3.72	0.0405	19	0.71	0.0920
J.	1.10	4.00	0.0630	62	2.47	0.0816
Wa.	1.58	4.08	0.0630	58	2.37	0.0664
T.	0.86	4.22	0.0900	104	4.41	0.0945
K.	1.03	4.33	0.1050	91	3.96	0.1065
Bo.		4.85	0.0980	89	4.32	
P.		4.89	0.0732	51	2.52	

TABLE II.

The 3rd Day of a Diet Constant in Nitrogen and Water. Groups of Cases in Which the Concentration of Urea in the Urine Was the Same.

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Urine concentration per 100 cc.	Blood concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's first law.
		gm.	*gm.*	*cc.*	*gm.*	
K.	1.03	1.00	0.0216	93	0.93	0.0450
C.	1.06	1.00	0.0210	75	0.75	0.0482
Bo.	0.81	1.27	0.0261	73	0.93	0.0614
K.	1.03	1.28	0.0246	56	0.72	0.0585
We.	1.13	1.25	0.0180	42	0.52	0.0482
K.	1.03	1.31	0.0216	43	0.57	0.0578
M.	1.09	1.34	0.0156	29	0.39	0.0491
C.	0.93	1.71	0.0246	56	0.96	0.0533
F.	0.90	1.74	0.0240	42	0.73	0.0605
Wi.	1.08	1.72	0.0246	34	0.58	0.0634
Add.	1.05	1.71	0.0276	28	0.48	0.0795
Ch.	1.09	1.73	0.0252	26	0.45	0.0737
Bo.	0.81	1.87	0.0300	47	0.88	0.0724
Wa.	1.58	1.90	0.0250	32	0.61	0.0520
McC.	1.19	1.95	0.0246	50	0.97	0.0468
S.	1.08	2.00	0.0240	30	0.60	0.0610
Ad.		2.21	0.0438	43	0.95	
P.		2.21	0.0282	30	0.67	
D.	1.00	2.29	0.0312	38	0.87	0.0684
Ma.		2.31	0.0210	22	0.51	

The 6th Day of the Same Diet with 20 Gm. of Urea in 360 Cc. of Water Taken on the 4th, 5th, and 6th Days.

C.	0.93	3.15	0.0624	87	2.74	0.0805
D.	1.00	3.23	0.0651	80	2.59	0.0828
Bo.	0.91	3.22	0.0590	72	2.32	0.0830
Cr.	1.06	3.21	0.0558	70	2.25	0.0737
K.	1.03	3.15	0.0672	68	2.14	0.0925
Add.	1.05	3.56	0.0678	69	2.46	0.0861
Ad.	0.99	3.54	0.0750	68	2.41	0.0991
F.	0.90	3.65	0.0558	66	2.41	0.0774
G.	0.95	3.63	0.0612	52	1.89	0 0935
Bo.	0.81	3.72	0.0672	58	2.16	0.0920
Ai.	1.19	3.69	0.0690	58	2.14	0.0885
J.	1.01	4.00	0.0630	62	2.47	0.0816
Wa.	1.58	4.08	0.0630	58	2.37	0.0664

TABLE III.

Groups of Cases in Which the Concentration of Urea in the Blood Was the Same.

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Blood concentration per 100 cc.	Urine concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's second law.
		gm.	*gm.*	*cc.*	*gm.*	
K.	1.03	0.0216	1.00	92	0.93	72.5
W.	1.13	0.0210	0.94	82	0.77	64.1
A.	1.06	0.0210	1.00	75	0.75	60.4
K.	1.03	0.0216	1.31	43	0.57	51.0
M.	1.09	0.0210	2.31	22	0.51	68.2
V.	0.95	0.0225	0.63	151	0.96	54.8
V.	0.95	0.0225	1.16	64	0.74	57.6
McC.	1.19	0.0246	1.95	50	0.97	122.2
C.	0.93	0.0246	1.71	56	0.96	88.9
B.	1.11	0.0240	1.06	87	0.93	80.6
F.	0.90	0.0240	1.74	42	0.73	65.7
K.	1.03	0.0246	1.28	56	0.72	63.6
S.	1.08	0.0240	2.00	30	0.60	69.6
Wi.	1.08	0.0246	1.73	34	0.58	62.5
T.	0.86	0.0252	3.15	48	1.51	175.0
J.	1.01	0.0252	1.62	52	0.84	82.0
Wa.	1.58	0.0250	1.90	32	0.61	101.0
Br.	1.01	0.0252	2.40	20	0.48	57.1
Ch.	1.09	0.0252	1.73	26	0.45	48.9
Mo.	0.82	0.0271	2.42	44	1.07	103.5
V.	0.95	0.0270	1.17	51	0.60	46.9
Add.	1.05	0.0276	1.71	28	0.48	50.0
Ai.	1.19	0.0282	2.07	39	0.81	105.0
Pa.		0.0282	2.21	30	0.67	
G.	0.94	0.0288	1.14	34	0.39	29.9
Ca.	0.84	0.0292	1.45	90	1.31	100.5
Sh.	1.00	0.0292	1.73	27	0.47	46.9
Add.	1.05	0.0300	0.18	732	1.14	38.6
Add.	1.05	0.0300	0.64	143	0.91	58.0
Bo.	0.81	0.0300	1.87	47	0.88	74.0
Add.	1.05	0.0300	2.06	18	0.37	42.4

TABLE III—*Continued.*

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Blood concentration per 100 cc.	Urine concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's second law.
		gm.	*gm.*	*cc.*	*gm.*	
Add	1.05	0.0312	0.18	596	1.08	36.6
D	1.00	0.0312	2.29	38	0.87	100.0
Add	1.05	0.0315	1.95	20	0.39	43.1
Boy	0.91	0.0324	0.15	760	1.18	31.5
Add	1.05	0.0322	1.29	80	1.03	93.2
Add	1.05	0.0322	1.91	45	0.86	94.5
Add	1.05	0.0322	0.77	112	0.85	59.5
Add	1.05	0.0322	1.47	44	0.64	61.8
Add	1.05	0.0322	0.97	54	0.52	40.7
Sh	1.00	0.0330	1.65	37	0.61	59.5
Sh	1.00	0.0330	1.94	24	0.47	52.2
Add	1.05	0.0336	0.19	572	1.10	38.3
Boy	0.91	0.0336	0.18	584	1.04	30.5
K	1.03	0.0337	1.04	84	0.88	70.1
Add	1.05	0.0337	0.44	181	0.81	42.0
Sh	1.00	0.0337	1.77	23	0.41	42.0
Ca	0.84	0.0360	0.91	127	1.15	70.0
K	1.03	0.0360	1.71	47	0.80	81.9
Ca	0.84	0.0367	1.30	81	1.05	76.5
Add	1.05	0.0367	2.05	51	1.04	118.4
Ch	1.09	0.0379	1.35	121	1.36	130.8
Jo	1.14	0.0375	2.63	37	0.97	135.8
Ca	0.84	0.0384	0.20	882	1.81	51.5
T	1.01	0.0384	0.15	718	1.05	31.2
Mo	0.82	0.0384	0.20	508	1.00	27.9
So	0.98	0.0382	3.71	20	0.74	106.0
T	1.01	0.0390	0.14	750	1.09	31.2
Ca	0.84	0.0390	1.30	81	1.06	77.0
J	1.01	0.0398	0.20	590	1.17	40.2
Jo	1.14	0 0397	3.31	25	0.83	131.5
Jo	1.14	0.0397	3.29	23	0.76	119.0

TABLE III—*Continued.*

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Blood concentration per 100 cc.	Urine concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's second law.
		gm.	*gm.*	*cc.*	*gm.*	
Add.....	1.05	0.0408	0.20	688	1.35	48.1
Mo.....	0.82	0.0402	0.21	508	1.09	31.0
Mo.....	0.82	0.0402	0.22	496	1.08	31.5
Add.....	1.05	0.0402	0.16	600	1.04	33.2
Jo.....	1.14	0.0405	3.72	19	0.71	118.8
Add.....	1.05	0.0414	0.72	196	1.30	87.9
Add.....	1.05	0.0414	0.17	640	1.09	35.9
Add.....	1.05	0.0427	0.92	193	1.78	136.4
Add.....	1.05	0.0426	0.23	704	1.60	61.2
So.....	0.98	0.0427	2.64	40	1.06	128.0
So.....	0.98	0.0427	3.51	27	0.94	131.0
Add.....	1.05	0.0427	2.23	41	0.91	108.1
So.....	0.98	0.0427	3.82	21	0.80	116.2
Ch.....	1.09	0.0435	2.61	68	1.77	236.5
Mo.....	0.82	0.0438	0.52	292	1.52	68.0
Mo.....	0.82	0.0435	2.68	38	1.02	104.0
Ad.....	0.99	0.0438	2.21	43	0.95	106.0
Ca.....	0.84	0.0450	0.20	922	1.81	67.0
Add.....	1.05	0.0450	2.33	17	0.40	48.7
Mo.....	0.82	0.0462	2.04	78	1.60	142.2
Add.....	1.05	0.0465	2.01	60	1.21	137.0
Add.....	1.05	0.0473	0.92	193	1.78	136.3
Add.....	1.05	0.0472	1.58	53	0.84	84.0
Add.....	1.05	0.0489	0.20	600	1.21	43.1
Mo.....	0.82	0.0485	2.22	48	1.07	99.4
Add.....	1.05	0.0495	1.99	104	2.06	231.8
Add.....	1.05	0.0495	2.38	71	1.70	209.5
Add.....	1.05	0.0514	2.53	79	2.00	253.8
B.....	1.11	0.0516	2.23	84	1.87	235.9
Add.....	1.05	0.0517	0.92	193	1.78	136.1
O.....	1.09	0.0546	2.34	110	2.58	329.8
Add.....	1.05	0.0540	1.95	51	1.00	111.3

TABLE III—*Concluded.*

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Blood concentration per 100 cc.	Urine concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's second law.
		gm.	*gm.*	*cc.*	*gm.*	
M	1.08	0.0558	1.43	216	3.09	304.1
F	0.90	0.0558	3.65	66	2.41	316.0
Cr	1.06	0.0558	3.21	70	2.25	324.0
S	1.08	0.0606	2.87	72	2.07	287.8
Add	1.05	0.0607	2.75	69	1.90	251.6
G	0.94	0.0612	3.63	52	1.89	258.0
C	0.93	0.0624	3.15	87	2.74	344.0
J	1.01	0.0630	4.00	62	2.47	379.5
Wa	1.58	0.0630	4.08	58	2.37	570.0
Bo	1.03	0.0672	3.72	58	2.16	326.0
K	1.03	0.0672	3.15	68	2.14	296.8
Add	1.05	0.0678	3.56	69	2.46	370.2
Add	1.05	0.0682	2.78	78	2.17	289.0

TABLE IV.

Groups of Cases in Which the Concentration of Urea in the Blood Was the Same.

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Blood concentration per 100 cc.	Urine concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's second law.
		gm.	*gm.*	*cc.*	*gm.*	
K	1.03	0.0216	1.00	92	0.93	72.5
Cr	1.06	0.0210	1.00	75	0.75	60.4
K	1.03	0.0216	1.31	43	0.57	51.0
Ma		0.0210	2.31	22	0.51	
McC	1.19	0.0246	1.95	50	0.97	122.2
C	0.93	0.0246	1.71	56	0.96	88.9
B	1.11	0.0240	1.06	87	0.93	80.6
F	0.90	0.0240	1.74	42	0.73	65.7
K	1.03	0.0246	1.28	56	0.72	63.6
S	1.08	0.0240	2.00	30	0.60	69.6
Wi	1.08	0.0246	1.73	34	0.58	62.5
T	0.86	0.0252	3.15	48	1.51	175.0
J	1.01	0.0252	1.62	52	0.84	82.0
Wa	1.58	0.0250	1.90	32	0.61	101.0
Br	1.01	0.0252	2.40	20	0.48	57.1
Ch	1.09	0.0252	1.73	26	0.45	48.9
Ai	1.19	0.0282	2.07	39	0.81	105.0
P		0.0282	2.21	30	0.67	
Add	1.05	0.0276	1.71	28	0.48	50.0

The 6th Day of the Same Diet with 20 Gm. of Urea in 360 Cc. of Water Taken on the 4th, 5th, and 6th Days.

We	1.13	0.0510	2.57	84	2.16	297.0
B	1.11	0.0516	2.23	84	1.87	235.9
Me	1.09	0.0558	1.43	216	3.09	304.1
F	0.90	0.0558	3.65	66	2.41	316.0
Cr	1.06	0.0558	3.21	70	2.25	324.0
S	1.08	0.0606	2.87	72	2.07	287.8
Br	1.01	0.0600	5.14	35	1.80	313.0
C	0.93	0.0624	3.15	87	2.74	344.0
T	1.01	0.0630	4.00	62	2.47	379.0
Wa	1.58	0.0630	4.08	58	2.37	570.0
Add	1.05	0.0678	3.56	69	2.46	370.2
Bo	1.03	0.0672	2.16	58	2.16	326.0
K	1.03	0.0672	3.15	68	2.14	296.8

TABLE V.

Groups of Cases in Which the Concentration of Urea in the Urine and in the Blood Was the Same.

Subject.	Weight factor, $\frac{70 \text{ kg.}}{\text{Kg. of body weight.}}$	Urine concentration per 100 cc.	Blood concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.
		gm.	*gm.*	*cc.*	*gm.*
Ca.....	0.84	0.20	0.0384	882	1.81
Mo.....	0.82	0.20	0.0384	508	1.00
Mo.....	0.84	0.21	0.0402	508	1.09
Mo.....	0.84	0.21	0.0402	496	1.08
K.....	1.03	1.00	0.0216	93	0.93
Cr.....	1.06	1.00	0.0210	75	0.75
C.....	0.93	1.71	0.0246	56	0.96
Ch.....	1.09	1.73	0.0252	26	0.45
F.....	0.90	1.74	0.0240	42	0.73
Wi.....	1.08	1.72	0.0246	34	0.58
Mc.....	1.19	1.95	0.0246	50	0.97
S.....	1.08	2.00	0.0240	30	0.60
Mo.....	0.82	2.04	0.0462	78	1.60
Add.....	1.05	2.01	0.0465	60	1.21
Ch.....	1.09	2.61	0.0435	68	1.77
Mo.....	0.82	2.68	0.0435	38	1.02
Jo.....	1.14	3.31	0.0397	25	0.83
Jo.....	1.14	3.29	0.0397	23	0.75
J.....	1.01	4.00	0.0630	62	2.47
Wa.....	1.58	4.08	0.0630	58	2.37

THE INCREASE OF NITROGEN IN FERMENTING MANURES.*

By W. E. TOTTINGHAM.

(From the Wisconsin Agricultural Experiment Station, Madison.)

(Received for publication, January 17, 1916.)

Various investigations which have been made concerning the changes of composition in fermenting stored farm manure have nearly always shown a greater or less loss of nitrogen. Such investigations usually have covered periods of fermentation ranging from 2 to 3 months to several months in length. As far as the writer is aware, very few investigators[1] have observed a gain of nitrogen.

In the course of an investigation of the changes in a fermenting mixture of cow and horse manure, with and without litter in the form of barley straw, the writer found gains in the absolute amount of nitrogen after 2 weeks and after 4 weeks of fermentation. Later there were decreases of nitrogen of such extent that after 12 weeks of fermentation the manures showed losses in the absolute amount of nitrogen present. The gain of nitrogen in this experiment was much greater in the straw-littered manure than in the control, unlittered manure. This phenomenon has received further attention, with the results reported in this paper. Acknowledgment is made of the necessary bacteriological assistance rendered by Dr. E. B. Fred of this Experiment Station.

The check experiment was conducted in the usual manner, as follows: Fresh cow manure and fresh horse manure, each free from any but fine litter, were mixed in the ratio of 2: 1. Chopped wheat straw was sifted into two portions one of which passed

* Published with the permission of the Director of the Wisconsin Experiment Station.

[1] Löhnis, F., Handb. landw. Bakteriol., Berlin, 1910, 745. Henry, *J. agr. pratique*, 1897, lxi, 411 and 485, Abstracts. Hornberger, *Centr. Bakteriol., 2te Abt.*, 1905, xiv, 423; 1907, xix, 340, Abstracts.

through a sieve of $\frac{5}{16}$ inch mesh, but was retained by a sieve of $\frac{1}{4}$ inch mesh. The other portion contained the straw which passed through the finer of the two sieves mentioned. Three portions of the mixed manure amounting to 6.25 kg. each were weighed. Lot 1 was left untreated as a control, Lot 2 received 0.5 kg. of the coarser straw litter, and Lot 3 received an equal amount of the finer straw. Each lot of manure was next thoroughly mixed and sampled for chemical and bacteriological analysis. It was then weighed in a loosely covered pail of galvanized iron and set aside in a warm room. The weighings were performed on a solution scale sensitive to 1 gm., or to within a possible error of 0.02 per cent for the weight involved. After a period of 4 weeks each lot of manure was weighed again, thoroughly mixed, and sampled as before for analysis. The data of the analyses follow:

TABLE I.

		Fresh weight.	Dry matter.		Loss of dry matter.	Nitrogen in dry matter.		Gain (+) or loss (−) of nitrogen.	Bacteria in millions per gm. of dry matter.
		gm.	*per cent*	*gm.*	*per cent*	*per cent*	*gm.*	*per cent*	
Lot 1. Control, manure alone...	At beginning.....	5,489	20.3	1,115	—	1.67	18.60	—	5.0
	After 4 wks.	5,281	17.1	904	18.92	1.95	17.63	−5.22	110.2
Lot 2. Manure with coarser wheat straw...	At beginning.....	6,095	25.0	1,524	—	1.38	21.03	—	53.5
	After 4 wks.	5,710	18.2	1,039	31.82	2.12	22.03	+4.76	167.0
Lot 3. Manure with finer wheat straw...	At beginning.....	6,118	25.2	1,540	—	1.40	21.56	—	25.1
	After 4 wks.	5,756	18.5	1,064	30.91	2.10	22.36	+3.71	210.5

The data show that there was an appreciable gain of nitrogen in the straw-littered manures. The gain was not so great as that

which had been obtained in the previous experiment in which barley straw had been used. It may be stated that the determinations of dry matter were made on triplicate samples of 100 gm. each, nitrogen was determined in either triplicate or quadruplicate by the Gunning method on 10 gm. samples of the moist manures, and the bacterial counts were made upon agar media.

The different manures were next tested for nitrogen fixation in mannite solution. It was not possible to identify *Azotobacter* forms among the organisms in the manures. Chromogenic forms were predominant among the bacteria which developed on agar plates from the straw-littered manures. The nature of the or-

TABLE II.

	Nitrogen.	Average.	Relative amount of nitrogen with control as 100.
	mg.	*mg.*	
Control, uninoculated.	Flask A, 1.1 " B, 1.0	1.05	100
Lot 1. Inoculated by extract of manure alone.	" A, 0.5 " B, 0.6	0.55	52
Lot 2. Inoculated by extract of manure with coarser wheat straw.	" A, 8.3 " B, 12.1	10.20	970
Lot 3. Inoculated by extract of manure with finer wheat straw.	" A, 12.7 " B, 10.5	11.60	1,100

ganism or organisms responsible for the fixation observed here is receiving attention under the direction of Dr. Fred.

Quantitative measurements of the fixing power of these manures were made by both weak and strong inoculations. The former was conducted as follows: 20 gm. of manure sample were diluted with 400 cc. of sterile water and 10 cc. portions of the dilution were inoculated into two 100 cc. portions of Ashby's solution in large Erlenmeyer flasks. Two similar flasks were treated immediately with 10 cc. of concentrated sulfuric acid, to serve as controls. After 3 weeks' incubation the eight flasks thus derived were analyzed for total nitrogen with the results given in Table II.

The results agree with those of the fermentation, showing the presence of nitrogen-fixing forms in the littered manures. The strong inoculation was conducted as follows: 10 gm. samples from each lot of manure were added to each of four flasks of Ashby's solution. Two flasks of each lot were immediately treated with 10 cc. of concentrated sulfuric acid. The twelve flasks were then incubated for 4 weeks and the contents analyzed for total nitrogen with the results given in Table III.

TABLE III.

	Nitrogen.	Average.	Gain in nitrogen in unacidified cultures.	
	mg.	*mg.*	*mg.*	*per cent*
Lot 1. Manure alone.				
Acidified.	Flask A, 33.7			
	" B, lost.	33.7		
Unacidified.	" A, 45.5			
	" B, 44.8	45.2	11.5	34.0
Lot 2. Manure with coarser wheat straw.				
Acidified.	" A, 38.2			
	" B, 37.7	38.0		
Unacidified.	" A, 51.0			
	" B, 48.3	49.7	11.7	31.0
Lot 3. Manure with finer wheat straw.				
Acidified.	" A, 39.0			
	" B, 39.3	39.2		
Unacidified.	" A, 49.2			
	" B, 47.3	48.3	9.1	23.0

The results show that the manures contain nitrogen-fixing organisms of considerable activity. As their activity was greatest in the fermentation experiments in those manures which were mixed with straw the latter material appears to be an important source of energy for the organisms.

It is apparent that the gain of nitrogen by manures in the early stages of fermentation may assume practical importance by the production of increased growth in crops unless it is counterbalanced by the loss of organic matter which occurs simultaneously. Through the courtesy of Professor E. B. Hart of this laboratory the writer is privileged to use data from field plots to investigate this

point. Manure is applied to these plots once during a three course rotation of corn, barley, and clover, application preceding the corn crop. Data covering 6 years show 5.3 per cent greater yield of corn grain and 7.4 per cent greater yield of stover from stall manure with straw as compared with fresh manure with straw. This difference disappears with the barley crop and is reversed with the clover crop. The stall manure is taken from the same supply as the fresh manure, but is allowed to stand in a covered box out of doors during the winter months, so that fermentation is limited.

From the results of this limited number of crops it is evident that increased value of the manure has resulted from restricted fermentation, the nitrogen so fixed becoming available during the season of application. Further work is necessary to determine how generally applicable the principle may become in the practical use of farm manure.

THE DETERMINATION OF CHOLESTEROL IN BLOOD.

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The probable importance of a knowledge of the variations in the cholesterol content of the blood in pathological conditions has been recognized for some time; and recently the variations of cholesterol in the normal blood in relation to its function in normal metabolism have been receiving attention. The earlier methods put forward for the determination of cholesterol in blood were gravimetric, requiring relatively large amounts of blood and considerable expenditure of time, and were therefore unsuited to the study of changes in the cholesterol content of the blood of the living organism. The more recent methods[1]—mainly colorimetric—are better suited to the purpose, since they require only a few cc. of blood and can be carried out in a short time. Of these methods the one which appears to have given the most general satisfaction is that of Autenrieth and Funk,[1] a colorimetric method depending on the Liebermann-Burchard color reaction. In this method 2 cc. of blood or plasma are digested for 2 hours on the water bath with strong alkali, the cholesterol is extracted from the digestion mixture by repeated shaking with ether or chloroform, and determined colorimetrically by treatment in chloroform solution with acetic anhydride and sulfuric acid. Aside from the long digestion, the inconvenient feature of this method is the extraction, which requires a total of 25 to 30 minutes of constant hard shaking together with five or six separations. These extractions when performed by hand are very tedious. Other objections to the method as carried out are: (1) the difficulty of ex-

[1] For a discussion of the methods for cholesterol determination in blood the reader is referred to Autenrieth, W., and Funk, A., *Münch. Med. Woch.*, 1913, lx, 1243.

tracting cholesterol from strongly alkaline aqueous solutions, which was pointed out by Corper;[2] and (2) the fact that the extract frequently contains something which gives a brownish tint to the green color produced in the chloroform and renders the readings difficult. The occurrence of the brownish tint was admitted by Autenrieth and Funk who claimed, however, that it did not interfere with the readings. Klein and Dinkin[3] noted the occurrence of the brown tint and state, on the contrary, that it does interfere with the determination. They ascribe the tint to the action of the strong alkali.

In earlier work on blood lipoids it has been found that the extraction of the lipoid material from the blood by treatment with alcohol-ether, as previously described,[4] is practically complete, and since the time required is short and the treatment relatively gentle it was believed that it offered a good means for the determination of cholesterol. Attempts were therefore made to apply the Autenrieth-Funk procedure to the determination of cholesterol in this extract. The procedure as finally worked out, while yielding higher results than that of Autenrieth and Funk, is much more simple and rapid and is therefore believed to be a considerable improvement over the latter.

An attempt was first made to use the untreated alcohol-ether extract of the blood. It was evaporated to dryness, the cholesterol taken up with chloroform, and the color produced as usual. The chloroform extract was, however, so much tinged with brown that the readings were always doubtful. To obviate the color difficulty the blood extract was next saponified by evaporation to dryness with sodium ethylate and the cholesterol extracted from the dry material by boiling out with several portions of chloroform. But although the extracts so obtained were colorless, the time required was so great that very little advantage was gained. It was then discovered that if the evaporation of the untreated alcohol-ether extract was carried out carefully so as to avoid heating the residue after it had reached dryness, the chloroform extract was colorless and after treatment gave excellent readings.

[2] Corper, H. J., *J. Biol. Chem.*, 1912, xi, 27.
[3] Klein, W., and Dinkin, L., *Z. physiol. Chem.*, 1914, xcii, 302.
[4] Bloor, W. R., *J. Biol. Chem.*, 1915, xxiii, 317, and earlier papers.

Method.

Preparation of the Sample.—3 cc. of whole blood, plasma, or serum are run slowly (a slow stream of drops) from a pipette into about 75 cc. of a mixture of redistilled alcohol and ether (3 parts alcohol, 1 part ether) in a 100 cc. graduated flask. The contents of the flask should be kept in motion during the process so that there is no clumping of the precipitated material. The contents of the flask are raised to boiling by immersion in a water bath (with constant shaking to avoid superheating), cooled to room temperature, filled to the mark with alcohol-ether, mixed, and filtered. The filtered liquid if placed in a tightly stoppered bottle in the dark will keep unchanged for a considerable time so that if it is not convenient to complete the determination at once, the sample may be carried to the above stage and left till a more suitable time.

By running the blood slowly into the large quantity of alcohol-ether, as above, the protein material is precipitated in finely divided form and under these conditions the short heating combined with the great excess of solvent is adequate for complete extraction of serum or plasma. The extraction, while not quite so complete in the case of whole blood, is believed to be better (because of the higher values obtained) than that obtained by any other method in use at the present time.

Determination.—10 cc. of the alcohol-ether extract are measured into a small flat-bottomed beaker and evaporated *just* to dryness on a water bath or electric stove. Any heating after dryness is reached produces a brownish color which passes into the chloroform and renders the subsequent determination difficult or impossible. The cholesterol is extracted[5] from the dry residue by boiling out three or four times with successive small portions of chloroform and decanting into a 10 cc. glass-stoppered, graduated cylinder which has previously been calibrated. The combined extracts after cooling (5 cc. or less) are then made up to 5 cc. The solution should be colorless but not necessarily clear, since the slight turbidity clears up on adding the reagents.

[5] In order to get an adequate extraction with the small amounts of chloroform used, an excess (3 or 4 cc.) should be added each time and the mixture allowed to boil down to half its volume or less, before decanting.

5 cc. of a standard cholesterol solution in chloroform[6] (containing 0.5 mg. of cholesterol) are measured into a similar 10 cc. cylinder.

To each of the solutions are added 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid, the solutions mixed by inverting several times, then set away in the dark for 15 minutes, after which they are transferred to the cups of the colorimeter (Duboscq) and compared as usual, setting the standard at 15 mm. The cement of the colorimeter cups must, of course, not be soluble in chloroform; plaster of Paris has been found satisfactory, or even ordinary glue if the cups are not used for any other purpose. In using the colorimeter in this determination the window screen described by Folin and Denis[7] has been found valuable. The error of the above method when carried out with ordinary care is 4 to 5 per cent. If greater accuracy is desired it may be obtained, at the expense of more material and time, by using 50 cc. of the alcohol-ether extract, evaporating as above, extracting with larger quantities of chloroform, making the extracts to 25 cc., and taking an aliquot of 5 cc. for the determination.

RESULTS.

Cholesterol added to alcohol-ether extracts of blood was recovered quantitatively. Parallel determinations were carried out with blood plasma by the Autenrieth-Funk method (ether extraction) and by the adaptation described above. The results are summarized in the table.

The results obtained by the new procedure are always higher (7 to 30 per cent, average 20 per cent) than by the Autenrieth-Funk method. Also the colors produced in the chloroform extract by the new procedure are almost invariably of the same tint as the standard, in marked contrast to those by the Autenrieth-Funk procedure, which almost always have the yellowish or brownish tinge mentioned above. In many cases this discolora-

[6] It is convenient to make the cholesterol standard in two strengths: (a) the stock solution containing 0.2 gm. of cholesterol (Kahlbaum) in 200 cc. chloroform; and (b) the standard solution for use, made by diluting 10 cc. of the above to 100 cc. with chloroform. 5 cc. of this latter solution will contain 0.5 mg.

[7] Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xviii, 263.

Plasma samples.	Cholesterol per 100 cc.	
	Autenrieth-Funk method.	New procedure.
	mg.	*mg.*
Dog I	180	210
" II	120	135
" III	141	147
" IV	100	130
Pig I	94	117
" II	80	104
Sheep	75	95
Rabbit	31	42
Human (carcinoma)	216	240
" (syphilis)	112	140
" (nephritis)	200	250

tion was so strong as to make the readings doubtful. Possible explanations for the higher results by the new procedure are: (1) the previously mentioned difficulty of extracting cholesterol from a strongly alkaline solution; and (2) changes in the cholesterol produced by the action of the strong alkali during the long digestion at high temperature in contact with the oxygen of the air. Autenrieth and Funk state, however, that further extraction produces no higher results, which would indicate that the second was the more probable explanation. That cholesterol is not entirely stable under conditions similar to the above has been pointed out by Lifschütz.[8]

[8] Lifschütz, J., *Z. physiol. Chem.*, 1906–07, l, 436.

MASS ACTION IN THE ACTIVATION OF UNFERTILIZED STARFISH EGGS BY BUTYRIC ACID.

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INTRODUCTORY.

In experiments performed at Woods Hole in the summer of 1914 I found that unfertilized starfish eggs, after treatment for several minutes with weak solutions of butyric acid in sea water (N/260), not only formed fertilization membranes on return to normal sea water, but cleaved more or less regularly and developed into freely swimming larvæ (blastulæ and gastrulæ).[1] In order to induce favorable development by this means the exposure must be several times longer than that normally allowed for artificial membrane formation; otherwise the treatment is the same; the eggs are simply returned from the acid-containing solution to normal sea water after a definite time in the solution, and receive no further treatment. In favorable experiments the great majority (80 to 90 per cent) of eggs thus activated formed larvæ; in similar experiments performed during the past summer the proportion of active and well developed larvæ has frequently reached 95 per cent or more. This treatment thus forms a highly favorable parthenogenetic method with starfish eggs; it does not, however, succeed with sea urchin eggs, which require a supplementary treatment (*e.g.*, with hypertonic sea water) to induce complete activation. In the experiments of Aug. 31 and Sept. 1, 1914, the optimal exposures varied from 6 to 10 minutes; briefer exposures of 2 to 5 minutes caused typical membrane formation, but cleavage was imperfect or delayed, and development usually stopped short of the swimming stage; with still briefer exposures of 1 minute

[1] Lillie, R. S., *Biol. Bull.*, 1915, xxviii, 260.

or less the eggs merely formed membranes and broke down without development. The effect of underexposure is thus to induce an activation which is imperfect or partial; in order to complete this activation all that is needed is to prolong the exposure sufficiently; the same result may be gained by treating the underexposed eggs a second time, after an interval in sea water, with the same fatty acid solution for an appropriate period.[2] There is thus a well defined optimum duration of exposure to the activating solution; exposures of less than this optimum are insufficient for complete activation; exposures prolonged a few minutes above the optimum injure the eggs, and development ceases early; with still longer exposures the eggs fail to cleave or even to form membranes.

The effects of higher temperatures on unfertilized starfish eggs (*i.e.*, of exposure to sea water at 30–35°) vary in an essentially similar manner with the duration of exposure; at each temperature a certain minimal exposure is required to induce membrane formation (at 32° about 2 minutes); one several times longer causes complete activation (about 8 minutes at 32°); while with still longer exposures (more than 12 minutes at 32°) the eggs fail to develop. The precise time of exposure required to produce each of these effects decreases rapidly as the temperature rises, being approximately halved by a rise of 1°.[3] Varying the time of exposure to warm sea water has thus the same physiological effect as similarly varying the exposure to a fatty acid solution,—a fact indicating that with both agents the essential condition underlying activation is the same.

The question arises as to the nature of this condition. It is to be assumed that the primary effect of exposure to the high temperature or to the fatty acid solution is to initiate some chemical process in the egg—probably an interaction between two substances already present in the protoplasm; this process has a critical or determinative relation to the normal sequence of physical and chemical changes constituting development. The above time relations indicate that in order to bring the egg into a con-

[2] Lillie, *Biol. Bull.*, 1915, xxviii, 291. After-treatment with hypertonic sea water, cyanide, or warm sea water (32–34°) may also be used to complete the activation.

[3] Lillie, *Biol. Bull.*, 1915, xxviii, 278 and 279, Tables IX and X.

dition in which it is capable of continuing automatically its development to an advanced stage, this critical interaction must proceed until a definite quantity of reaction product has been formed; it is to be presumed that the reaction product is then present in its appropriate situation at a certain definite concentration. If this concentration is not reached activation is only partial, and only a few of the early steps of development are carried out, as seen in the formation of the fertilization membrane, and the execution of some cleavages of less than normal rate; if it is exceeded the egg is also rendered incapable of prolonged development, though for a different reason. That the failure of development after membrane formation by brief exposure to the activating condition is due simply to an insufficient formation of this reaction product, is indicated by the fact that a second treatment, either with fatty acid or high temperature, completes the activation and enables the egg to form larvæ. No such treatment will restore the power of development to eggs that have been overexposed to either agent.[4]

It seems clear that some single process, comparatively simple in its nature, is started by the activating agent; this process is progressive and requires time, and in order to induce normal development it must proceed to a certain stage and then be arrested. The nature of the chemical factor in the activation process cannot be inferred with definiteness from the above data; some light, however, appears to be thrown on the condition under which the critical combination takes place. When unfertilized eggs are brought into sea water at 30°, the activation process is initiated and continues progressively as long as the egg is kept at this temperature, reaching its completion in about 30 minutes; at 32° the same stage of activation is reached in 7 to 8 minutes, at 34° in 3 minutes, and at 36° in 1 minute. The activation process thus exhibits a high temperature coefficient (Q_{10} = 200 to 400); this coefficient is similar in its order to that of the decrease of viscosity or degelation of colloidal systems of gelatin and water under the influence of rising temperature.[5] This fact suggests that the

[4] It is possible that the effects of overexposure may be reversible under other conditions, but this has not yet been determined.

[5] Compare von Schroeder, P., *Z. physik. Chem.*, 1903, xlv, 75; Levites, S. J., *Z. Chem. u. Ind. Kolloide*, 1907-08, ii, 211; also the discussion in the paper cited above, Lillie, *Biol. Bull.*, 1915, xxviii, 294.

direct effect of the high temperature upon the egg is to cause some change in the colloidal consistency of certain regions or structures, involving decrease of viscosity or degelation; this latter change permits the combination of constituents which in the resting egg are kept apart through their inability to diffuse. Under such conditions the rate of combination will be determined by the rate of diffusion (the reciprocal of viscosity), and hence will show the same temperature coefficient as the decrease of viscosity. The specific nature of the chemical combination involved is, however, not indicated by experiments with high temperature alone.

The fact that in the activation of eggs by butyric acid the same relations are found as in heat activation between time of exposure and physiological effect produced, suggests that the first chemical change in the activation process is a combination in which an acid takes part. It is evident, however, that any acid concerned in normal fertilization or heat activation cannot be a fatty acid, but must be one that diffuses less readily through the protoplasmic system; the possibility suggests itself that it may be an hydroxy-acid like lactic acid,—which moreover is known to be produced by stimulation in many protoplasmic systems. If the primary chemical event in activation is a chemical union between an acid and some other component of the egg system, and if this acid may be replaced by the rapidly diffusing butyric acid, the activating effect of butyric acid should be influenced by temperature and concentration in the manner characteristic of chemical reactions; *i.e.*, the rate of the activation process should be increased in a definite proportion by a given rise of temperature, or by a given increase in the concentration of the acid.

The question is whether butyric acid activates the starfish egg by combining chemically with some protoplasmic constituent, *i. e.*, by forming a compound like an ester, or by altering some physical condition, such as the state of the colloids at the egg surface, thus indirectly enabling another and critical combination to take place. The low concentration and brief exposure required for the action indicate that a direct alteration of the colloids is not the primary effect produced by the acid. On the other hand, the differences observed by Loeb[6] in *Strongylocentrotus* eggs with fatty

[6] Loeb, J., *Biochem. Z.*, 1909, xv, 254; Artificial Parthenogenesis and Fertilization, Chicago, 1913, 133.

acids, hydroxy-acids, and mineral acids, indicate that all acids are alike as regards the possession of activating power; the rate of penetration is the chief factor determining action in any particular case. Loeb and Hagedoorn also found that the least effective duration of exposure to butyric acid solution was halved by raising the temperature from 10° to 20°,[7] also indicating that the acid acts by chemical combination. If this is the case, the minimal time of exposure ought also, according to the mass action law, to be halved (at constant temperature) by doubling the concentration of the acid; but a proportionality of this kind does not appear to exist in *Strongylocentrotus* eggs.[8] The conditions in this egg are, however, more complex than in the starfish egg; acid causes only partial activation (membrane formation) which has to be completed by an appropriate after-treatment; *e.g.*, with hypertonic sea water. Bases as well as acids may also cause membrane formation in sea urchin eggs;[9] the membrane-forming effect would thus appear in this egg to have no direct relation to the chemical nature of the agent employed. In the starfish egg the conditions differ in two essential respects: first, the activation following the proper exposure to butyric acid is complete; and second, this effect cannot be produced by a typical base like ammonia. The complete lack of any activation effect with bases, as contrasted with the completeness of the effect produced by acid, seems especially significant. This negative action is illustrated by the following experiment. On June 14 the unfertilized eggs from several starfish were exposed to solutions of ammonium hydroxide of the following concentrations: 0.0005 N, 0.001 N, 0.0015 N, 0.002 N. Van't Hoff's artificial sea water was used to avoid the disturbing effects of carbonates. Eggs were placed in each of these solutions at about 20° during the early maturation period, between the breakdown of the germinal vesicle and the separation of the first polar body, and were returned to normal sea water at intervals of 1, 2, 3, 4, 6, 8, 10, 12, 15, 18, 21, 25, 30, 35, and 42 minutes. The results were uniformly negative; in no case was there membrane formation or cleavage; all eggs

[7] Loeb, Artificial Parthenogenesis and Fertilization, Chicago, 1913, 146.
[8] Loeb, Artificial Parthenogenesis and Fertilization, Chicago, 1913, 135.
[9] Loeb, Artificial Parthenogenesis and Fertilization, Chicago, 1913, 147; *J. Exp. Zool.*, 1912, xiii, 577.

died without signs of development, as in the untreated control. The fertilized control eggs showed normal development. The range of concentrations and exposures employed is amply sufficient to prove the ineffectiveness of this base as activating agent. In *Strongylocentrotus* eggs, on the other hand, solutions of ammonia and other weak bases in about N/1675 concentration (0.3 cc. 0.1 N base plus 50 cc. sea water) were found by Loeb to be highly effective membrane-forming agents. It seems clear, therefore, that in the *Asterias* egg bases and acids differ entirely in their relation to the activation process; and since both butyric acid and ammonia are lipoid-soluble compounds and penetrate the egg readily, it is to be inferred that the contrast in their physiological effects is determined by the difference in their general chemical properties; *i.e.*, that acids act by combining chemically with some protoplasmic constituent and not by their physical action on the colloids.

Experiments on the relative rates of action of butyric acid solutions of constant concentration on starfish eggs at different temperatures have not yet been tried. Experiments at constant temperature with different concentrations of butyric acid indicate clearly that a direct relation exists between concentration and rate of action. With eggs of uniform condition and a range of concentrations of 0.00075 N to 0.006 N it was found that the time of exposure required for activation varied inversely with the concentration, showing a close approach to the linear relation required by the law of mass action. This comes out most clearly in a series of experiments performed during early June (Table III), at which time the condition of the starfish eggs at Woods Hole is most uniform and favorable. The same relation was also found later in the summer (July and August), although the eggs were then less regular in their behavior and responded more rapidly to the action of the acid; *i.e.*, the effective exposures for a given concentration were shorter.

EXPERIMENTAL.

In the following experiments the eggs were placed in the solution of butyric acid during the early maturation period, before the separation of the first polar body, and after the time interval indicated they were returned to normal sea water. The condition

of the eggs and the proportion developing to larval stages were afterwards determined. In the earlier series the solutions of butyric acid were made by adding the 0.1 N solution to sea water. In the later experiments the butyric acid solutions were made in van't Hoff's artificial sea water, to avoid the disturbing influence of neutralization.

The essential results of this treatment are the same with all effective concentrations of acid; the briefest exposures induce membrane formation, which may be normal, but is followed by imperfect cleavage and early breakdown; as exposure is prolonged more and more above this minimum the proportion of cleaving and favorably developing eggs increases progressively, reaching a maximum with a certain time of exposure which is shorter the more concentrated the solution; with further increase of exposure the proportion of eggs reaching larval stages again declines; cleavage becomes slower and development ceases sooner; eventually even fertilization membranes fail to form. A partial or imperfect activation is readily induced; with any given concentration of acid a relatively wide range of exposures may thus be effective for inducing membrane formation or the first few cleavages; but in order to insure the development of all normal eggs to larval stages the time of exposure to the solution must be definite. It is noteworthy that the rate of cleavage is faster and the cleavage itself more regular the nearer the exposure approaches the optimum.[10] In the case of any solution of butyric acid, the manner in which the degree of activation varies with varying times of exposure may be represented by a curve with the percentage of eggs forming larvæ as ordinates, and times of exposure as abscissæ; this curve has an approximately symmetrical form;[11] its range as measured along the abscissæ is shorter the higher the concentration. In the case of heat activation the curve relating the duration of warming (*e.g.*, at 32°) to the proportion of eggs forming larvæ has a similar form.[12] The time range occupied by this curve decreases with rising temperature,

[10] Lillie, *Biol. Bull.*, 1915, xxviii, 283, Table XII.

[11] Lillie, *Biol. Bull.*, 1915, xxviii, 282.

[12] See Lillie, *Biol. Bull.*, 1915, xxviii, 279, Table X, for a summary of the data.

just as that of the curve of acid activation decreases with rising concentration of butyric acid.

Table I gives a summary of all of last summer's experiments in which eggs were exposed for varying times to solutions of butyric acid in sea water. The 0.1 N butyric acid was added to sea water as indicated (0.001 N means 1 cc. of 0.1 N butyric acid plus 99 cc. of sea water). The carbonates of sea water partly neutralize the acid added, and the 0.001 N and 0.002 N solutions are for this reason ineffective; the other solutions all cause membrane formation and development with sufficient times of exposure. The duration ranges from 1 to 25 minutes; in each series eggs were transferred from the acid solutions to sea water at regular intervals,—of 1 minute with the briefer exposures; of 2 to 3 minutes with the longer ones. The table gives in each series the range of exposures used and the optimum duration of exposure, together with the approximate proportion of eggs forming larvæ at this exposure. In some cases this optimum was not reached.

The optimum exposure thus decreases progressively with increase in the concentration of butyric acid. With the six effective solutions the respective optima are: 0.003 N, doubtful (more than 30 minutes); 0.004 N, about 30 minutes; 0.005 N, 12 to 15 minutes; 0.006 N, 8 to 12 minutes; 0.007 N, about 6 minutes; 0.008 N, about 5 minutes. The relation between time of exposure and concentration is not constant in this series, as seen in the difference between 0.004 N and 0.005 N; but the results with artificial sea water indicate that this divergence is due simply to the removal of part of the butyric acid by neutralization; if a correction is applied for this effect an approximately constant inverse proportionality is seen. Acid added to 0.002 N concentration is completely, and to 0.003 N in greater part neutralized, as regards its physiological effect on the eggs; if we assume that with the remaining solutions the only effective acid is that present in excess of 0.003 N, the following results are obtained (Table II), which agree closely with those obtained a few days later with artificial carbonate-free sea water (Table III). The product of corrected concentrations and effective times of exposure should, according to the law of mass action, be a constant. Table II indicates that this is approximately the case; these results should be compared with Table IV, which gives the results of similar experiments with artificial sea water.

TABLE I.

Date.	Concentration	Range of exposures.	Results.
	N	*min.*	
May 31	0.001	3–12	No membranes; eggs die without change.
" 31	0.002	3–12	" " " " " "
" 31	0.003	3–12	All eggs form membranes; with 12 min. exposure 10–20 per cent form larvæ; optimum not reached.
June 2	0.003	1–15	15–20 per cent form larvæ with 15 min. exposure; optimum not reached.
May 31	0.004	3–12	30–40 " " " " " 12 " " " " "
June 2	0.004	1–15	35–40 " " " " " 15 " " " " "
" 4	0.004	3–25	65–75 " " " " " 25 " " " " " probably ± 30 min.,
" 2	0.005	1–15	Optimum at 12 min., with ± 85–90 per cent larvæ.
" 4	0.005	3–25	" " 14–17 " " ± 90 " " " At 20 min., 70–80 per cent; at 25 min., 30–40 per cent.
" 4	0.006	3–25	Optimum at 10–14 min., with 80–90 per cent larvæ At 17 min., 25–30 per cent.
" 5	0.006	1–20	" " 7–8 " " 70–80 " " " " 10 " 50–60 " " At 17 min., 5–10 per cent.
" 5	0.007	1–20	No larvæ with 1–2 min.; optimum at 6 min., 90–95 per cent larvæ; at 10 min., 30–35 per cent; none at 14 min.
" 5	0.008	1–20	At 2 min. 1–2 per cent form larvæ; optimum at 5 min. (> 90 per cent); at 7 min., 35–45 per cent; none at 10 min.

During the week of June 7 to June 13 similar experiments were carried out with solutions of butyric acid in van't Hoff's artificial sea water (100 volumes 0.5 M NaCl + 7.8 vol. 0.5 M $MgCl_2$ + 3.8 vol. 0.5 M $MgSO_4$ + 2.2 vol. 0.5 M KCl + 2 vol. 0.5 M $CaCl_2$), using the concentrations 0.0005 N, 0.00075 N, 0.001 N, 0.0015 N, 0.002 N, 0.0025 N, 0.003 N, and 0.004 N. All these solutions cause membrane formation and activation; with the 0.0005 N solution exposures of less than 4 minutes proved insufficient for membrane formation, and the longest exposures (42 minutes) caused only 4 to 5 per cent of the eggs to develop to larvæ; with all the other solutions the optimum exposure was reached, at which 90 per cent or more of the eggs formed larvæ. The effect of varying the duration of exposure was the same in all cases;

TABLE II.

Concentration of acid as added.	Corrected concentration of acid (C).	Optimum time of exposure (T).	Product ($C \times T \times 10^3$).
N	N	*min.*	
0.001	0		
0.002	0		
0.003	?	? >30	
0.004	0.001	±30	±30
0.005	0.002	12–17	24–34
0.006	0.003	8–12	24–36
0.007	0.004	±6	24
0.008	0.005	±5	25

the briefest effective exposures induce typical membrane formation, followed by irregular change of form and breakdown; with longer exposures the proportion and rate of development increase progressively up to a well defined optimum, after which there is a second decline.

The results of these experiments are indicated in Table III. The optimum duration of exposure with each solution, and the proportion of eggs forming larvæ are given as before. The solution of butyric acid in van't Hoff's sea water was added to a small volume of eggs; at intervals eggs were transferred by pipettes to normal sea water.

Throughout these experiments the control eggs showed uniformly normal behavior, practically all undergoing maturation

TABLE III.

Date.	Concentration.	Range of exposures.	Results.
	N	*min.*	
June 13	0.0005	1–42	Only 4–5 per cent form larvæ with 42 min. exposure; optimum not reached.
" 13	0.00075	1–42	80–90 per cent form larvæ with 42 min. exposure; this exposure is probably about the optimum; no longer exposures were made.
" 7	0.001	3–25	Longest exposure, 25 min.. gave only 1–2 per cent larvæ; optimum not reached.
" 8	0.001	3–20	Only 1–2 per cent larvæ with 20 min. exposure; optimum not reached.
" 13	0.001	1–42	Optimum at 35 min. with about 95 per cent larvæ; at 42 min. rather less favorable, 80–90 per cent.
" 10	0.0015	1–20	40–50 per cent larvæ with 20 min. exposure; optimum not reached.
" 13	0.0015	1–42	Optimum at 20–25 min. with ± 95 per cent larvæ; at 30 min., 20–25 per cent.
" 7	0.002	3–25	" " ±14 " " 80–90 " " " " 17 " ±65–70 " "
" 10	0.002	1–20	" " 15 " " 70–80 " " " " 20 " 65–75 " "
" 11	0.0025	1–20	" " 12 " " >95 " " " " 14 " ±90, at 17 min., 35–40 per cent.
" 7	0.003	3–25	Optimum at 7–10 min. with 80–90 per cent larvæ with each exposure at 7, 8, and 10 min.; at 14 min., ± 40–50 per cent.
" 11	0.003	1–20	Optimum at 10 min., with > 95 per cent larvæ: 8 and 12 min. also favorable, with 80–90 per cent; at 14 min., 30–40 per cent.
" 7	0.004	3–25	Optimum at 6–7 min., with ± 85–90 per cent; 8 min. is only slightly less favorable, with ± 75–80 per cent; at 10–12 min., 10–15 per cent.

and developing normally after fertilization; the above results may therefore be regarded as typical. Later in the summer the condition of the eggs was more variable, and the optimum time of exposure to a given solution proved decidedly shorter,—a change of condition due probably to a combination of factors (higher temperature, approaching end of breeding season, etc.). Experiments carried out later in the season are described in Table V.

In Table IV I have summarized the results of all the experiments in which a large majority of eggs (80 per cent or more) formed swimming larvæ with the optimal times of exposure. Experiments performed with different solutions upon the same lot of eggs are placed together under the same date. Usually some

TABLE IV.

Date.	Concentration (C).	Optimum time of exposure (T).	Product (C×T×10⁴).
		min.	
June 7	0.002	±14	±28
" 7	0.003	7–10	21–30 (average 25.5)
" 7	0.004	6–7	24–28 (" 26)
" 10	0.002	±15	±30
" 11	0.0025	12–14	30–35 (average 32.5)
" 11	0.003	8–12; 10 best	24–36 (" 30)
" 13	0.00075	±42	±31.5
" 13	0.001	±35	±35
" 13	0.0015	20 and 25	30–37.5 (average 34)

variation in the condition of the eggs is observed from day to day, but at this time of year such changes are comparatively slight; later they may become considerable, as Table V shows.

These results show that with solutions ranging from 0.00075 N to 0.004 N the product of concentration and optimal time of exposure is an approximate constant for each solution, with an average value of about 30. In other words, the velocity of the activation process—the change within the egg enabling it to continue development to a larval stage—is proportional to the concentration of the butyric acid. This fact furnishes strong evidence that the acid acts by combining chemically with some constituent of the egg system, and that the presence of a definite

quantity of reaction product, which must not be exceeded, is the prerequisite for complete activation.[13]

Other experiments carried out later in the summer yielded a similar result, but the value of the CT product was in all cases lower than the above; *i.e.*, the eggs become more sensitive to the activating influence of the acid; the experimental results also show greater variability. The results are summarized in Table V, which includes all those experiments in which a large proportion (50 per cent or more) of mature eggs formed swimming larvæ. The experiments were all performed between July 28 and Aug. 9, except the first two (June 28 and July 1); in these the eggs appear to show an intermediate condition.

From July 28 to Aug. 3 the relation between duration of effective exposure and concentration of acid shows some irregular fluctuation, but on the whole it remains fairly constant; the average value of the product CT during this period is about 12; this is less than half the value observed in early June (about 30); from Aug. 4 the product is again higher (average about 19) and resembles that of the two series of June 28 and July 1. The condition of the starfish eggs at this time of year, near the end of the breeding season, is variable, and perhaps a greater constancy is not to be expected. The reciprocal of the product CT may be regarded as a measure of the sensitivity of the eggs to the acti-

[13] These results might conceivably be interpreted as indicating merely that the essential requirement for activation is the entrance of a certain minimal quantity of acid into the egg, and that the rate of entrance is a direct function of the concentration gradient between the medium and the cell interior. If, however, the simple rate of entrance is the essential factor determining the above relation between concentration and rate of activation, we should expect that the effective times of exposure would be only slightly affected by temperature. In the above cited experiments of Loeb and Hagedoorn with *Strongylocentrotus* eggs the time required for membrane formation was found to be halved by raising the temperature from 10° to 20°. I have not yet investigated the influence of temperature on the rate of acid activation in the starfish egg. In general it seems probable, from the rapidity with which lipoid-soluble substances enter cells, that the time required for simple entrance in the above experiments is negligible in comparison with the time required to produce the physiological effect within the egg. If the rate of this activation effect is proportional to the concentration of the activating agent, and shows the chemical temperature coefficient, the presumption seems fair that the agent acts by combining chemically with some cell constituent.

vating influence of the acid; *i.e.*, as a coefficient of sensitivity. What determines these variations of sensitivity is uncertain; during the warmer part of the summer CT showed a lower value than earlier or later; *i.e.*, the eggs responded then most quickly to the acid; the change on Aug. 4 seems to correspond with a change in the weather which at that date turned decidedly cooler. I have not as yet made definite experiments on the influence of tem-

TABLE V.

Date.	Concentration (C).	Range of exposures.	Optimum (T).		Result of exposure next above optimum.	Product ($C \times T \times 10^3$).
		min.	*min.*	*per cent*	*min.* *per cent*	
June 28	0.003	1–17	6	90	At 7 and 8, 70–80.	18.0
July 1	0.003	1–17	7–8	80–90	" 10, 20–30	21–24
" 28	0.005	0.5–4	2	±95	" 2.5, 80–90; at 3, ±50	10.0
" 28	0.006	0.5–4	1.5	90–95	" 2, ±50	9.0
" 29	0.0035	1–10	3	70–80	" 4, 50–60	10.5
" 29	0.0045	1–10	2	65–70	" 3, 10–15	9.0
" 30	0.0035	1–6	2.5–3	85–90	" 3.5, 65–75	8.8–10.5
" 30	0.0045	1–6	1.5–2	70–85	" 2.5, 25–35	6.8–9.0
" 31	0.003	1–9	2–2.5	85–90	" 3, ±50	6.0–7.5
" 31	0.004	0.5–4.5	1	70–80	" 1.5, 35–45	4.0
Aug. 1	0.003	0.5–5.5	3	80–90	" 3.5, ±50	9 0
" 1	0.004	0.5–5	1.5	40–50	" 2, 2–3	6.0
" 3	0.003	0.5–5	4–4.5	80–90	" 5, 75–85	12.0–13.5
" 3	0.004	0.5–5	2.5–3	85–90	" 3.5, 65–75	10–12.0
" 4	0.002	1–12	9–10	80–90	" 12, 75–85	18–20
" 7	0.002	1–14	9	85–90	" 10, 65–75	18.0
" 7	0.0025	1–14	7–9	80–90	" 10, 60–70	17 5–22.5
" 9	0.0015	2–22	12–14	55–65	" 16, 15–20	18.0–21.0

perature on this property of the eggs.[14] Internal factors, such as variations in the physiological conditions of the animals at dif-

[14] I have found, however, that the presence of various chemical substances in the medium may greatly alter the time of exposure required for activation, either by high temperature or fatty acid solution. This is illustrated by the following experiment. On July 1 eggs were exposed to 0.003 N butyric acid dissolved (*a*) in unaltered van't Hoff's solution, and (*b* and *c*) in van't Hoff's solution containing respectively 3 and 4 volumes per cent ethyl alcohol. The optimum time of exposure in the absence of alcohol (*a*) was 7 to 8 minutes (70 to 80 per cent larvæ); in both the alcohol-containing solutions the optimum was about 3 minutes (80 to 90 per cent larvæ), and somewhat shorter in *a* than in *b*; 5 minutes was well past the

ferent periods of the breeding season, are probably more important. Of chief interest is the fact that at the height of the season, early June, when the eggs are most abundant and most uniform in behavior, CT had a value of approximately 30 and showed relatively slight variation. This condition probably represents the norm in these eggs.

SUMMARY.

1. Unfertilized starfish eggs may be caused to develop into larvæ by a sufficiently prolonged exposure to weak solutions of butyric acid in sea water or van't Hoff's solution. No supplementary or "corrective" treatment is required.

2. In order to produce complete activation the eggs must be exposed to the solution for a certain minimal time which is a function of the concentration of acid. Exposures briefer than this optimum cause partial activation,—membrane formation followed by imperfect cleavage and breakdown; activation may be completed by a second exposure to the same solution. Exposures longer than the optimum injure the eggs and interfere with activation.

3. Activation is a progressive process which under uniform conditions of temperature and physiological state of the eggs appears to proceed at an approximately uniform rate. This rate is closely proportional to the concentration of butyric acid, within a range of 0.0005 N to 0.006 N.

4. The most consistent interpretation of these facts seems to be that the acid activates the egg by combining chemically with some egg constituent until a certain quantity of a definite reaction product is formed. The time required to produce this critical quantity will, in accordance with the mass action law, be inversely proportional to the concentration of acid.

optimum, while brief exposures (2 minutes) produced a considerable number of larvæ (in *b* 10 to 15 per cent; in *c* 20 to 30 per cent), in contrast to *a* where an exposure of 4 minutes was required to produce 5 to 10 per cent larvæ. The whole activation process thus proceeds from two to three times more rapidly in the presence of alcohol than in its absence. Alcohol alone has no activating effect; it merely promotes or facilitates the action of the fatty acid,—sensitizes the egg, as it were. Alcohol similarly shortens the effective time of exposure to warm sea water. The basis of such effects is uncertain as yet; but they show that the rate at which the activation process proceeds depends on certain modifiable physicochemical conditions in the egg system. I hope to consider this subject at greater length in a separate paper.

THE PRESENCE OF EPINEPHRIN IN HUMAN FETAL ADRENALS.

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A number of investigators have found epinephrin in considerable amounts in the adrenals from fetuses of different animals, by both chemical and physiological methods. Some of the same investigators and others have been unable to detect it in human fetal adrenals. Moore and Purinton[1] analyzed human fetal adrenals by the $FeCl_3$ and blood pressure methods with negative results. Švehla,[2] using the blood pressure method, did not find epinephrin in the human fetus, but found it in the adrenals of the dog fetus. Langlois and Rehns[3] were able to show pressor substances in sheep fetal adrenals. Fenger[4] found large amounts of epinephrin in the adrenals of beef fetuses, and McCord[5] obtained physiological reactions in the sixth week of gestation in cattle. Cannata[6] did not examine the adrenals, but he tested the serum of healthy new-born infants for epinephrin. His results were negative.

It is difficult to understand the differences in the physiology of the fetuses of man and the lower animals that would explain why one should possess epinephrin and the other not. Suspecting that the disparity in the findings of previous investigators might lie in the use of faulty methods, a series of analyses of human adrenals was begun. Recently Folin, Cannon, and Denis[7] have

[1] Moore, B., and Purinton, C. O., *Am. J. Physiol.*, 1900–01, iv, 57.

[2] Švehla, K., *Arch. exp. Path. u. Pharm.*, 1900, xliii, 321.

[3] Langlois, J. P., and Rehns, J., *Compt. rend. Soc. biol.*, 1899, li, 146.

[4] Fenger, F., *J. Biol. Chem.*, 1912, xi, 489.

[5] McCord, C. P., *J. Biol. Chem.*, 1915, xxiii, 435.

[6] Cannata, S., *Pediatra*, 1915, xxiii, 244.

[7] Folin, O., Cannon, W. B., and Denis, W., *J. Biol. Chem.*, 1912–13, xiii, 477.

described a chemical method for determining epinephrin in tissues, which is both simple and delicate. A reagent is used which consists of a mixture of phosphoric acid and sodium tungstate. The reagent gives a blue color with solutions containing epinephrin. Uric acid also gives the same color, but this is, for the same amount, three times more intense and is therefore used as a standard in titrating tissues for epinephrin. This test seemed admirably adapted for the problem undertaken. Several investigators have used it in other work and have reported it to be satisfactory.

The method of proceeding was to obtain the fetus as fresh as possible. The adrenals were taken out, dissected free of adventitious tissue, weighed, and ground up in 0.1 N HCl and H_2O with sand. The mixture was boiled with the addition of sodium acetate according to the directions of Folin and Denis. After being made up to a standard volume, the extract was tested with the phosphotungstate reagent. The blue color produced was compared with that produced by 1 mg. of uric acid. The results are seen in Table I.

TABLE I.

"Epinephrin" Content of Fetal Adrenals as Determined by the Folin-Denis Colorimetric Method.

Age of fetus.	Weight of two adrenals.	Amount of "epinephrin" in both glands.
mos.	*gm.*	*gm.*
3	0.304	0.00058
3	0.222	0.00029
3	0.464	0.00033
5	1.825	0.0004
7	1.515	0.00033
Full term, still birth.	2.34	0.00066
Full term, still birth.		0.00166

If these determinations represent the actual amounts of epinephrin in the adrenals, the amounts are large enough to be detected by other methods. Therefore another series of analyses was begun with the blood pressure method, Seidell's manganese dioxide test,[8] the $FeCl_3$ test, and the $AuCl_3$ test.

[8] Seidell, A., *J. Biol. Chem.*, 1913, xv, 197.

All these tests were negative when made on a series of human fetal adrenals ranging from 3 months to full term. Boiled NaCl extracts of these adrenals constantly gave a marked fall in blood pressure and never an undoubted pressor effect. We are aware that extremely small amounts of epinephrin cause a fall in blood pressure instead of a rise. The injection of an extract of fetal liver or kidney will produce a similar depressor action, which is ascribed to the reaction with foreign proteins or the products of their cleavage. And possibly some of it is due to the action of choline. It would be impossible to determine if an additional fall of blood pressure due to small amounts of epinephrin was superimposed on the fall produced by simple tissue extracts.

It occurred to us that probably the reason for the finding by the Folin-Denis method of amounts of epinephrin in human fetal adrenals that could not be detected with other methods is that fetal tissues contain a relatively large amount of uric acid. Uric acid has been found in considerable quantities in the kidneys of the new-born, leading to the formation of the so called uric acid infarcts. Whether or not other fetal or infantile tissues contain much uric acid cannot be determined from the literature, because no reference can be found to analyses of such tissues for uric acid.

A 7 months fetus was obtained and the kidneys were removed. It was then ground up and analyzed by the Krüger-Solomon method for uric acid. 100 mg. were found. These results are striking because corresponding amounts of uric acid have not been found in normal adult human tissues. The adrenals from the same fetus and four other fetuses were ground up and extracted with 0.1 N HCl; the extract was analyzed physiologically for epinephrin and chemically for uric acid. The physiological test was negative and the chemical analysis showed 0.005 gm. of uric acid.

Uric acid accumulates in human tissues because they do not contain enzymes that can destroy uric acid. This is not true of most other mammals. In order to determine whether the Folin-Denis test agreed with the physiological test for epinephrin in tissues that do not contain such large quantities of uric acid, two sets of analyses were made of fetal beef adrenals. 53 gm. of adrenals were obtained from beef fetuses, unselected as to size. These were ground up and extracted with 0.1 N HCl. This extract

was titrated for epinephrin by the blood pressure method, with Parke-Davis adrenalin 1:3,200 as a standard. By this method 0.045 gm. of epinephrin was found. The Folin-Denis test gave 0.06 gm. Another lot of 193.5 gm. was obtained and analyzed by the same methods. The physiological test gave 0.087 gm. of epinephrin, and the Folin-Denis test gave 0.12 gm. Analyses of this lot by the Krüger-Solomon method for uric acid gave 0.003 gm., the presence of which may be accounted for by the fact that uricase is not formed until late in the development (Mendel and Mitchell[9]).

The most delicate test for epinephrin is the method in which an intestine or uterus strip contracting in oxygenated Ringer's solution is used. This method is reported to detect epinephrin in a dilution of 1:1,000,000. As a final experiment for the purpose of finding epinephrin in human fetal adrenals, the glands from a 6 months fetus were obtained 18 hours after death. The fetus had been kept on ice during this time. A boiled extract was made of the ground up adrenals in 6 cc. of Ringer's solution. A guinea pig was killed instantaneously by a blow on the head, and a piece of the small intestine about 3 cm. long was tied at one end to an L-shaped glass rod and to a sensitive lever at the other end, and then immersed in a tube containing warm Ringer's solution through which bubbled a slow stream of oxygen. The tube containing the strip was put into a large tank of water which was kept uniformly at 39°C. When contractions were well established the effects of adrenalin and the extract of the fetal adrenals were determined. These solutions were kept in the same bath with the strip and were introduced into the tube through an opening in the bottom. Adrenalin stopped the contractions and inhibited the tone promptly while the extract had no effect whatever.

The adrenals from two full term fetuses were next obtained and extracts of the ground up glands were made with Ringer's solution. One of the fetuses had lived a few seconds, and the other half an hour after birth. The extract of the former was tested with a strip of guinea pig intestine contracting *in vitro* under the same conditions described in the preceding experiment, and the extract

[9] Mendel, L. B., and Mitchell, P. H., *Am. J. Physiol.*, 1907–08, xx, 97.

of the latter tested with a strip of rabbit uterus and intestine. The tone and contractions of the two intestinal strips were inhibited, while the tone of the uterine strip was increased, indicating the presence of epinephrin in the glands of both fetuses. These experiments were sufficiently controlled by comparing the effect of adrenalin.

These last two experiments are the nearest to an actual demonstration of the presence of epinephrin in human fetal adrenals that we have obtained, yet they are not free from objections because such sensitive objects as intestinal and uterine strips contracting *in vitro* are apt to be not specific in their response to substances acting on them. Stewart[10] emphasizes that such tests should be controlled with other biological tests.

There is reason to suspect that epinephrin is contained in the human fetal adrenals. These glands in the fetus are strikingly large, in proportion to the size of the other organs, when compared with the adult adrenals, and there is no doubt that their size has some relation to their functional activity. As far as we know, the needs of the human fetus differ in no respect from those of the lower animals, and no reason suggests itself for the absence of epinephrin in one and its presence in the other. A possible explanation for the difficulty in demonstrating it in human fetal adrenals lies in the fact that a normal human fetus is rarely obtained. Spontaneous and surgical abortions are usually the result of some abnormal condition of the mother. Facts concerning prenatal pathology are meager and we know nothing of the influence of these conditions on the fetus. Usually the fetus is subjected to prolonged asphyxia during delivery which may influence the adrenals. It may also be that oxidative enzymes are more active in the fetus than in the adult, and that postmortem destruction of epinephrin is more prompt.

The Folin-Denis test is undoubtedly the best colorimetric test that has been devised for the detection of epinephrin, but, like all its predecessors, has its limitations. The experiments recorded in this paper make clear one of its most serious disadvantages. Just as it cannot be used to titrate epinephrin in fetal tissues, it ob-

[10] Stewart, G. N., *J. Exp. Med.*, 1912, xv, 547.

viously cannot be used with adult tissues from gouty individuals, because of their high content of retained uric acid.[11] Neither can it give accurate results in the assay of tissues from birds, because of their characteristic uric acid metabolism.

SUMMARY.

1. Epinephrin could not be found in human fetal adrenals by the use of the blood pressure method and chemical tests.

2. A suggestion of the presence of epinephrin was found in the adrenals of two full term fetuses by the use of strips of uterus and intestines contracting in warm oxygenated Ringer's solution. The extract of the adrenals of a 6 months fetus tested with a contracting intestinal strip was entirely negative.

3. The Folin-Denis test cannot be used to determine epinephrin in fetal tissues, because of their high content of uric acid.

[11] Fine, M. S., and Chace, A. F., *J. Biol. Chem.*, 1915, xxi, 371.

CELL PENETRATION BY ACIDS.

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Contributions from the Zoological Laboratory of the Museum of Comparative Zoology at Harvard College, No. 270, and the Bermuda Biological Station for Research, No. 41.

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INTRODUCTION.

A study of the chemoreceptors of the earthworm *Eisenia foetida*, begun at the suggestion of Professor G. H. Parker, resulted in the accumulation of data which could not be analyzed in terms of current ideas relating to cell permeability and its rôle in stimulation. At the same time it led to certain views regarding the problem of irritability; namely, that this question may best be studied with the sense organs of animals of moderate structural complexity, rather than in simple[1] protozoa or in relatively non-specialized cells. In a ceptor cell the processes of irritability stand forth with especial clearness, because less obscured by the course of other activities than is probably the case with the majority of tissue cells; irritability is so predominantly the function of the ceptor

[1] Dobell (1914, page 181) states: "It is safe to prophesy that when the known facts have been doubled or trebled, the ironical statement . . . . that 'the Protozoa are the simplest organisms in which to study the great problems of biology' will disappear from biological literature."

that it may here be studied in a reasonably pure condition.[2] Organs of chemical sense seem particularly well adapted for such experiments. Moderate morphological complication is a necessary restriction for technical reasons, since it is only with animals of not too high an organization that quantitative methods of experiment involving the measurement of responses may successfully be used.

Part of the study referred to dealt with stimulation by acids, and the facts observed in this connection were responsible for the present work on the penetration of cells by acids.

Previous to the recent paper of Harvey (1914), cell penetrability for acids had not been studied by any direct and approximately quantitative method, largely because, as Harvey remarks, cells containing indicators appropriate for this work have seldom been observed.[3] He was able to utilize the pigmented gonidial filaments of a holothurian, *Stichopus ananus* Jaeger, and measured the time required for the testis of this animal to change color when placed in 0.01 N solutions of a variety of acids. The results of his experiments with acids of this concentration led Harvey to the view that probably lipoid solubility, or capillary activity, and affinity for proteins of the cell surface were both factors in determining the particular penetration speed observed with a given acid, but that there was no agreement between penetration speed and ionization.

During the summer of 1913 I found that the blue pigment of a nudibranch, *Chromodoris zebra* Heilprin, gave an extract with dependable qualities as an indicator, and in a brief note (Crozier, 1914) I described some properties of the substance. It was also found that acids would change the color of the indicator within the cell.

[2] I have since observed that a similar idea has been developed by Lucas (1909, particularly page 328): "The physiology of the cell must begin, not with that maid-of-all-work, the unicellular organism, but with such cells as are as nearly as possible unifunctional." "The only practical method for the study of any function will be to investigate that function first in some cell in which it appears in a highly elaborated state."

[3] The statement is often made that indicators are of rare occurrence in animals, but I have discovered such materials during the last 3 years in such widely separated forms as: a balanoglossid, a common Bermudian and West Indian tunicate, and an annelid, in addition to the one herein described.

Harvey's list of acids, though embracing a number of substances, was studied at only one concentration, and previous work on stimulation by acids had convinced me that series purporting to indicate the relative physiological activity of acids were not likely to be of general value when constructed on such a basis. This expectation has been completely justified (Crozier, 1915). It is with this problem, the penetration rate of acids as a function of concentration, and the interpretation of stimulation by acids, that the present account is concerned. The conclusions to which I have come regarding permeability to acids are in a way similar to those of Harvey, aside from the part assigned to ionization, but the method of analysis is different and based upon more complete data.[4]

The Indicator.

The only characteristics of the *Chromodoris* indicator which are important here are (a) its mode of occurrence and (b) the influence of various conditions upon the magnitude and rapidity of its color changes. Some hints have been obtained as to the chemical nature of the material and its significance for the so called warning coloration of the animal, but these will be described in another place.

For example, this indicator gives positive results with nine tests for alkaloids, but otherwise gives no reaction with protein precipitants; it appears to be intimately connected with the animal's spicy odor and general immunity from enemies. The pigment appears to bear some relation to the blue material described by von Zeynek (1901, 1902) from the fish *Crenilabrus*. From the fact that the blue substance is decolorized on reduction and may be restored to its original hue by H_2O_2, some writers would doubtless regard it as a respiratory pigment.

(*a*) *Occurrence.*—The position of the pigment in the cell cannot be made out in sections, because it dissolves. Observations are therefore restricted to teased cells and tissues manipulated in a compressorium. Such observation shows that several forms of the pigment are present. Along the border of the mantle it can be seen that by far most of the material is situated at the base of the epithelial cells, in the form of sac-like bodies of variable

[4] The control of permeability to acids by salts, and other phases of this problem, are also being studied.

size and (apparently changeable) shape. But blue granules occur quite near to the outer surface, and the small spherical bodies which occur in the mucus on the animal's surface are strongly tinged with blue.

The coloration of *Chromodoris* depends upon the blue pigment and a yellow one. Along the border line between blue and yellow patches, cells can be made out which contain both yellow and blue pigment, like the doubly pigmented erythrophores of *Ostracion* described by Ballowitz (1913); but such cells are infrequent.

(*b*) *Color Changes.*—The yellow pigment is not soluble in water and cannot be extracted by aqueous alcohol, aqueous acetone, or pyridine. It is soluble in xylol, full strength alcohol, acetone, and other fat solvents. Its globules stain with dyes that color fat.

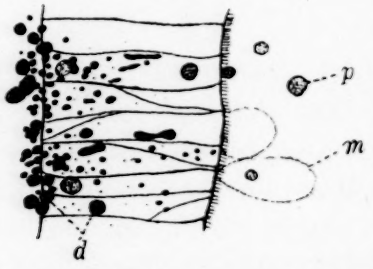

FIG. 1. From a camera sketch of a compressed preparation of the edge of the mantle. *d* = dense blue bodies; *p* = pale spherical masses; *m* = mucus. × 125.

The blue bodies indicated below the basement membrane were located in near-by cells of the epithelium, the outlines of which are not shown at this focus.

The blue substance, which is soluble in water, is the only indicator involved. Pyridine, a weak base, apparently extracts it in the least changed condition, the solution being blue or blue-green. Alcoholic or aqueous extracts are at first blue with a reddish fluorescence, but subsequently turn purple, with a strong blue fluorescence. As an indicator this pigment behaves somewhat like litmus, though its tints are more pronounced. Solutions of the pigment in fresh water or in 4 per cent solutions of formalin in either fresh water or sea water, and alcoholic extracts or aqueous solutions of the residue from an evaporated pyridine extract, give sensibly the same end-point when a few drops are added to 10 cc. of test solutions. The hydrogen-ion concentration for the

transition blue → pink, determined by phosphate and acetate mixtures, is the same, $p_H = 5.6 \pm$; this holds for solutions containing sea-water salts (NaCl + KCl + $CaCl_2$, at $\frac{5}{8}$ M total concentration), therefore corresponding presumably to the condition in the cell. 0.001 N solutions *in vitro* of the eighteen acids I have used effect the blue → pink transformation instantly. Dilute solutions containing the proteins of *Chromodoris* may give apparent end-points as low as $p_H = 4.7$.[5]

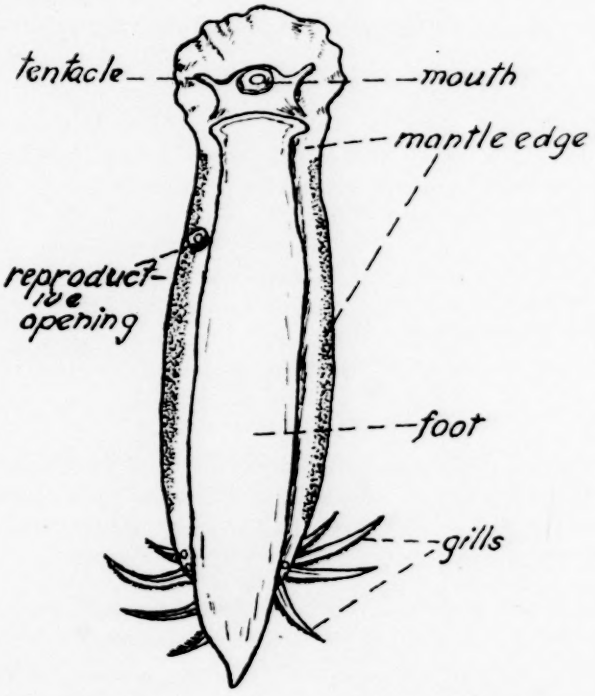

FIG. 2. *Chromodoris zebra*, diagrammatic ventral view; stippled areas show regions from which pieces were cut for the study of acid penetration. × ¾.

Method.

Pieces were cut from the lateral mantle edge of *Chromodoris* shown by Fig. 2. The animals were in every case freshly collected and in good condition, though I cannot agree with Smallwood (1910, page 140) that these animals do not well survive laboratory confinement. The pieces excised were about 10 mm.

[5] Compare Harvey (1913).

long by 5 to 6 mm. broad at the widest place, but tapering to a point at either end, and usually 2 to 3 mm. thick along the line of cutting, thinning rapidly to less than 0.5 mm. at the extreme outer edge. The penetrability of the different regions of

TABLE I.

Comparative Penetrability of Different Parts of Chromodoris to 0.01 N Acetic Acid. Time of Visible Penetration in Minutes.

	Penetration.
	min.
Rhinophores	1.0
Tentacles	1.5
Buccal mantle	4.0
Gills	4.0
Ventral surface of marginal mantle	75
Dorsal " " " "	>120
Foot. edge	100
Buccal margin of pharynx	35
Inner wall " "	90

TABLE II.

Penetration of Pieces Cut from the Midlateral Mantle of Individuals (Chromodoris) of Different Sizes. The Pieces Were All of the Same Size, Two or Three from Each Individual. 0.1 N HCl.

Animal.		Penetration. (27°C.)		
No.	Length.			
	cm.	*min.*	*min.*	*min.*
1	2.5	1.00	1.25	1.00
2	3.7	1.75	1.50	
3	3.9	1.75	2.00	2.25
4	5.5	1.00	1.25	1.20
5	6.2	3.00	3.25	
6	6.4	3.50	3.00	3.25
7	7.8	3.75	4.00	
8	10.4	2.75	3.50	3.25

an animal varies considerably (Table I), and for animals of different sizes the speed with which acids produce color change in pieces cut from corresponding locations is somewhat related to the length of the individual, as indicated in Table II, smaller speci-

mens tending to show greater penetrability. Consistent results were, however, easily obtained when these size and location factors were made uniform, and especially when the under surface of the mantle was employed.[6] Animals 8.5 to 10 cm. long were used.

The density of the blue coloration varies widely in different individuals, though the general scheme (Table I) is consistent for any one animal, as far as the area we are considering is concerned (Fig. 2). Material was therefore selected from animals showing a smooth blue pigmentation of the under surface of the mantle.

The source of error involved at this point lies less in the different metabolic conditions with which the several degrees of pigmentation may be correlated, and in the fact that the concentration of the indicator may affect its rate of color change by acids, than in one's inability to judge

TABLE III.

Penetration of Pieces of the Lateral Mantle by 0.01 N HCl (21°C.).

Coloration.		Penetration.				
		1	2	3	4	5
		min.	*min.*	*min.*	*min.*	*min.*
A	Purplish blue....	5.5	5.0	6.7	6.9	7.3
B	Blue.....	8.0	6.9	7.2	9.0	5.4
C	Spotted.....	8.9	9.6	7.5	8.1	7.3

accurately as to the exact point at which the pink hue becomes evident. *Chromodoris* shows four rather well defined conditions as regards the character of the blue mantle pigmentation; these may be termed the albino (see Smallwood, 1910, page 141), the blue, the purplish blue, and the spotted. The spotted condition is one in which the blue coloration is confined to circular dots less than 1 mm. in diameter, which are separated by distances usually of several mm., the intervening surface being pure white or slightly tinged with blue. These color varieties are not very clear-cut, the blue showing occasionally a spotting of denser color, but for convenience I have used them in Table III to show the variation in penetration time observed with the different modes of coloration.

Chromodoris is an hermaphrodite, and animals of the size we are dealing with reproduce at all times of the year; therefore sex

[6] Part of the pigment on the dorsal surface seems to be in a different physical or chemical condition, such that it is not easily dissolved out or changed in color.

and position in the reproductive cycle are of no consequence in the present connection.

The pieces to be tested were removed by a clean cut with sharp scissors while being held gently with forceps. They were then allowed to remain in sea water for about 10 minutes. As one result of the cutting there occurred a considerable secretion of slime. The pieces also contracted, in part through loss of the water which gives the tissue turgescence, and the edges of the cut surface began to roll inward over the plane of the cut. Immediately before transfer to the acid the pieces were washed in fresh sea water and carefully rolled between layers of clean filter paper; this wiping, which removed the slime and adherent sea water, was necessary to give uniform results, but control tests showed that treatments of this sort from one to five times in succession, allowing one-half minute in sea water between successive applications of the filter paper, did not affect the penetration time of the acids.

Each experiment consisted in immersing a piece of the *Chromodoris* tissue in a vessel[7] of thin colorless glass holding about 25 cc. of acid. The acid solutions were made up in recently caught rain water. Contact with the acid led to a further production of slime, the mucin of which was promptly coagulated. That the thin layer so formed did not affect the penetration of the acid was proved by experiments in which the film of mucin was carefully lifted off with forceps; this did not accelerate penetration to any measurable degree. At intervals of about a minute the solutions were stirred very gently. The volume of the pieces was 0.5 cc. ±, and it was found that this ratio of 1:50 between the volumes of tissue and solution was sufficient to preclude a decrease in acidity of the fluid during the longest time over which an experiment extended (120 minutes). The glass vessel was placed upon a slip of white porcelain, and the color changes were viewed in diffuse light from a northeast window.

Even after long practice it is necessary to have comparison standards, if a color change—even so distinct a one as that here employed—is to be judged promptly. For this purpose I have used two fresh pieces of tissue cut from the same animal and of the same size as the piece to be tested,

[7] Small crystallizing dishes were used.

one of the fragments (blue) being kept in normal sea water and the other in sea water recently made acid (pink) either by 0.01 N HCl or by some of the acid being tested. These controls were placed on the porcelain surface beside the piece whose color change was being timed.

The time elapsing between immersion in acid and the assumption of the pink color was taken with a stop-watch. In order to avoid color fatigue, the pieces of tissue were not viewed continuously but inspected at intervals of about a quarter of a minute. The point of transition from blue to pink could be determined with an accuracy indicated by a maximum error of 10 to 15 seconds. *It is important to note that the color change did not first*

TABLE IV.

*Data from One Experiment Comparing the Penetrability of Living and of Dead Cells (Experiment 56.1).**

Acid.	Control.	Dead cells.
	min.	*min.*
HCl, 0.1 N	2.2	1.2
	2.1	0.9
H_2SO_4, 0.05 N	5.0	2.5
	2.6	1.1
	2.5	1.0
Acetic, 0.02 N	20.0	9.0
	17.0	6.0

* The control pieces were cut from positions on the opposite side of the animal in the same relative location as the corresponding cells killed with 4 per cent formalin.

occur along the edge of the cut and from there progress laterally, but appeared evenly and with reasonable sharpness over the whole surface of the pieces studied. The blue pigment is more dense at the dorsal and ventral surfaces of the mantle than in the deeper tissues, but it could be seen that a faint pink hue was produced on the cut surface; this did not influence the development of the color transition on the ventral side.

A point of importance is the relation of the penetration rates of acids in the case of cells killed previous to immersion in acid. Killing by heat causes the coagulation of the pigment and the assumption of a green color; now, alkalies induce this reaction *in vitro*, the p_H value for the change being about 7.6 (at 27°), and

the effect of heat upon the indicator within the cell is to be ascribed to the increased alkalinity brought about by the thermal coagulation of proteins (Quagliariello, 1912). Cells killed by formalin do not give constant results, but the indication (Table IV) is (1) that cells so killed are more easily penetrated than those not previously killed, and (2) that the increased penetrability is not such as to make the cells readily permeable for all acids, though it amounts to about twice that of cells treated with acid while living.

Penetration Speed and Concentration.

Proceeding in the manner indicated by the previous section, the time of penetrations from a number of concentrations was determined for eighteen acids. The results are summarized in Table VII. Ten or more observations were made at each dilution. The general concordance of the separate measurements, which naturally is better at higher concentrations, may be illustrated by the records in Tables V and VI.

As I have elsewhere remarked (Crozier, 1915), the series of penetration rates from 0.01 N solutions shows a noteworthy correspondence with Harvey's (1914) measurements at the same dilution. The reasonable closeness of this agreement (Table VIII), considering that two widely separated animals were employed, and that in one case a portion of the integument and in the other an internal protected organ (testis) furnished the cells experimented upon, makes it logical to assume that a similar order of acid penetration is generally valid.

TABLE V.

*Penetration of Chromodoris Tissue by 0.01 N HCl (Experiment 10.13).**

No.	Penetration.	No.	Penetration.
	min.		*m n.*
1	9.0	6	8.0
2	7.0	7	8.25
3	8.0	8	6.9
4	7.5	9	7.25
5	5.5	10	8.5
	Mean.....		7.6±0.78

*A second series, 3 months later, gave 7.5±0.50.

TABLE VI.

Experiments with Oxalic, Monochloroacetic, and Acetic Acids.

No.	Penetration.		
	Oxalic, 0.002 N.	Monochloroacetic, 0.005 N.	Acetic, 0.01 N.
	min.	*min.*	*min.*
1	18.0	18.3	57
2	19.5	17.0	90
3	20.6	13.0	70
4	17.5	14.2	67
5	19.6	17.5	85
6	16.3	12.5	60
7	19.5	16.5	86
8	18.0	20.0	78
9	21.0	11.3	80
10	25.0	16.0	76
Mean	19.5 ±1.6	15.6 ±2.4	75 ±9.1

TABLE VII.

Acid.	Penetration time in minutes (2[illegible]°C.).																		
Normal dilution.	10	15	20	50	75	100	125	150	200	250	300	325	350	400	500	650	700	750	1,000
Hydrochloric	2.9			5.8	7.0	7.6				9.5					12.5			16.3	20.0
Sulfuric	2.7		3.2	5.9	7.5	8.5		10.5			13.5				17.0			24.7	
Oxalic	4.0			6.0	8.0	8.8				13.5					19.5	25.0			
Nitric	3.6			5.8		8.4				14.5			17.7		22.0		30.0		
Formic	1.75				4.0	4.5		9.2	13.0		15.0				24.5				
Salicylic				1.8	2.1	3.6	6.4	8.0	11.6	16.3	18.5	32.0			56.5				
Valeric (iso-)				1.2	1.5	1.9		6.0	12.0		26.0			55.0	70.0				
Monochloroacetic	5.6			7.4		10.0			15.5		24.0								
Malonic			4.7			10.0		15.2	22.5	24.0	36.0								
Benzoic						9.7		14.8		29.7									
Lactic	4.6			6.0	7.9	8.6	11.5		21.3	32.5									
Tartaric	5.5			8.8		13.5			32.0	60.0									
Malic	5.9		6.1			14.5			37.5	67.0									
Citric	7.0					16.0		26.0	40.0										
Succinic		6.9				16.5			45.0										
Butyric	7.6			12.6	15.0	19.0		30.0	50.0										
Propionic	7.1					30.0		55.0											
Acetic	11.9			19.5	30.5	75.0													

TABLE VIII.

Cell Penetration from 0.01 N Solutions.

Acid.	*Chromodoris*, mantle edge (27°).	*Stichopus*, testis (Harvey).
	min.	*min.*
Valeric (iso-)	1.9	2–4
Salicylic*	3.6	0.25
Formic	4.5	2–4
Hydrochloric	7.6	9–11
Nitric	8.4	
Sulfuric	8.5	
Lactic	8.6	
Oxalic	8.8	12–15
Benzoic*	9.7	0.25
Monochloroacetic*	10.0	2–4
Malonic	10.0	30
Tartaric	13.5	
Malic	14.5	40
Citric	16.0	
Succinic	16.5	—
Butyric	19.0	45–60
Propionic	30.0	
Acetic	75.0	

* The acids not corresponding in the two series are marked with an asterisk.

The curves of Fig. 3 show the relation between normality dilution (*i.e.*, liters containing one equivalent) and average penetration rate. They bring out features of the penetration process which measurements at a single dilution (Harvey, 1914) leave obscure. Series showing the relative penetrating power of acids vary with the concentration chosen (Crozier, 1915) and it follows that existing correlations of the mode of penetration for different acids with lipoid solubility, toxicity, and the like, must be revised. The form of the dilution curve as a whole must be used to determine the position of an acid in the penetration series.[8] On this basis the acids studied may be separated into two groups, an end also attained, though with less accuracy, by consideration of the limiting concentrations beyond which no color change is effected inside the cell.

[8] A consideration of this kind applies likewise to studies on the effects of acid solutions of equal hydrogen-ion content.

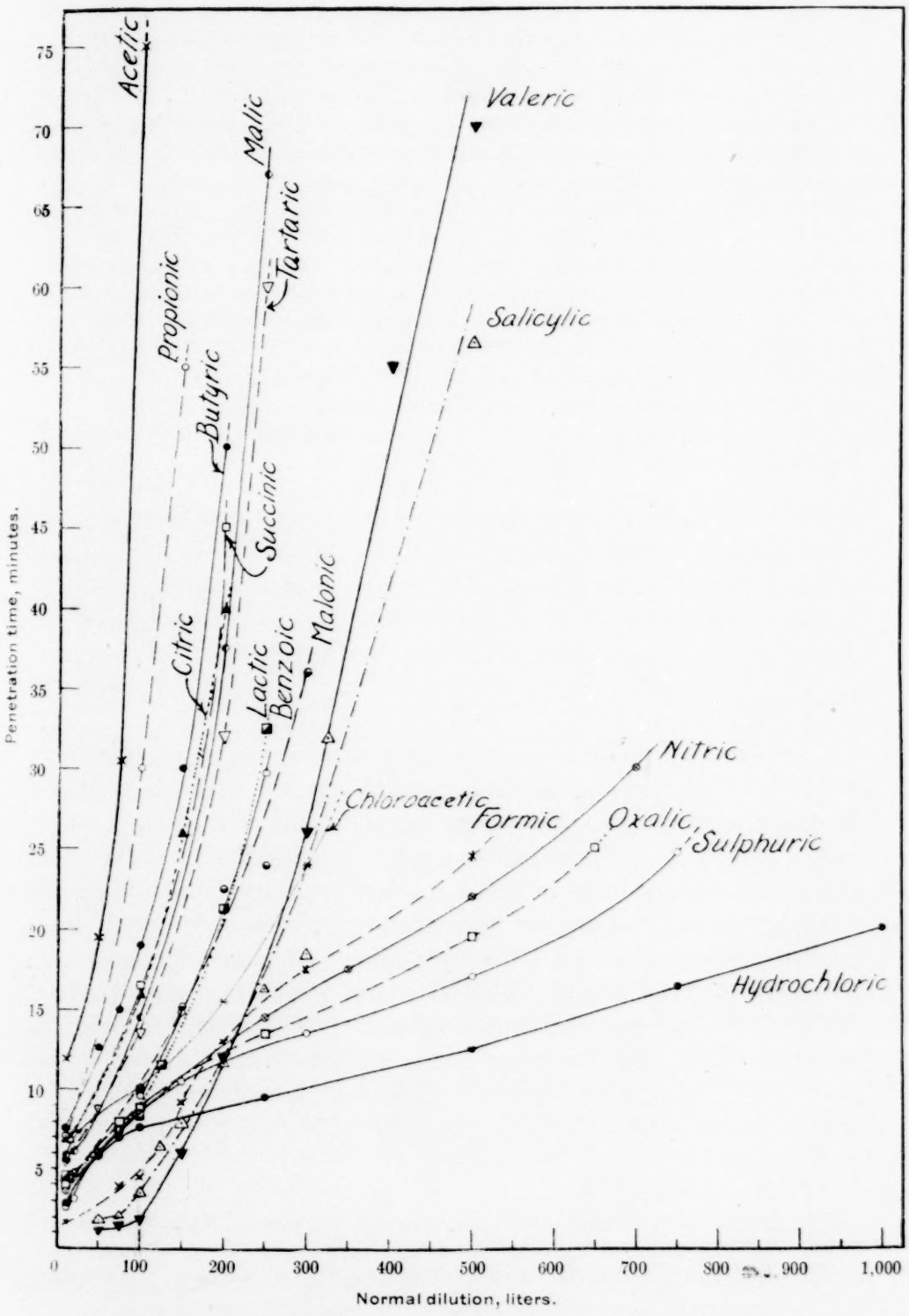

FIG. 3. The relation between dilution and time of penetration.

Outward diffusion of pigment occurs in acid solutions but this diffusion does not bear any constant relation to the time occupied by the internal color change, being variable and depending in part upon the degree to which the piece of tissue is contracted or passively stretched. The use of this outflow of pigment as an index of permeability changes is therefore not free from an objection which may be urged against much recent work on quantitative aspects of the permeability problem; namely, that cell pigment may be squeezed out in purely mechanical fashion, or that mechanical stresses may control the speed and extent of pigment loss. In general, with solutions giving rapid penetration, the spontaneous outward movement of pigment occurred after the color change within the cell; with lower concentrations, before an internal condition of acidity had been made visible. Moreover, as will be shown subsequently, it is possible experimentally to increase or decrease the penetration time of a given acid solution, and in certain of these cases the state of permeability as revealed by pigment diffusion is exactly the reverse of that shown by the speed of acid penetration.

The order of decreasing efficiency in penetration for the eighteen acids studied is approximately as indicated in Table IX, where position in the series is compared with ionization strength.[9]

At first sight there is little precise concordance between penetration speed and ionization, but from the tangle of curves the following sets of chemically related acids may be taken as probably significant.

1. The acids of high ionization are obviously set apart from the others by the character of their dilution curves, while as a whole the most weakly ionized acids are at the other end of the series; and I take the relative position of the members of these two groups to indicate that ionization is the primary determinant of cell penetrating power, but that some other factor[10] controlling entrance into the cell is also operative. On the one hand the ionization of HCl, H_2SO_4, $(COOH)_2$, and HNO_3[11] is so great that the second factor has relatively free sway in fixing their individual position, while, on the other hand, the ionization of the fatty acids is so weak that here also another factor determines the order of penetration, excepting that of formic acid, which is ioni-

[9] For the first $H^{\cdot}$.

[10] It is hardly probable, and certainly not necessary, that this second factor should be the same for different acids.

[11] Borowikow (1913) noted the order HCl > H_2SO_4 > HNO_3 > $(COOH)_2$ for the acceleration of plant growth.

cally stronger than the others (compare its different type of curve).

2. Among the weak fatty acids the order of penetration is: *valeric* > *butyric* > *propionic* > *acetic*; some clue to the mechanism of the process is clearly afforded by the fact that this is precisely the order of their action upon the surface tension of water and of their solubility in xylol (Harvey, 1914, page 948).[12]

TABLE IX.

Order of Penetration Compared with Ionization.

Acid.	Ionization constant.
Hydrochloric	(100.0)
Sulfuric	(100.0)
Oxalic	3.8
Nitric	(100.0)
Formic	0.0214
Salicylic	0.1020
Valeric (iso-)	0.0017
Monochloroacetic	0.155
Malonic	0.163
Benzoic	0.0060
Lactic	0.0138
Tartaric	0.100
Malic	0.0395
Citric	0.0870
Succinic	0.00665
Butyric	0.00153
Propionic	0.00137
Acetic	0.00187

3. Monochloroacetic acid is more highly ionized than acetic, and penetrates more easily.

4. With the saturated dibasic acids the order of penetration is: *oxalic* > *malonic* > *succinic*, which parallels exactly that of their ionization; and here again oxalic, the acid of much greater ioniza-

[12] This would appear to relate the activity of these acids to their lipoid solubility, a view favored by Harvey (1914) and, for other acids, by Vernon (1913), though on insufficient evidence. Their exact relation to the fatty substances of *Chromodoris* cells has not yet been determined.

tion than the other two, is set apart from them by the character of its curve.

5. Hydroxy substitutions, which result in increased ionization, give three clear-cut instances of increased penetrating ability likewise accompanying this change: (a) *lactic* > *propionic*, (b) *tartaric* > *malic* > *succinic*, (c) *salicylic* > *benzoic*.

There consequently seems no escape from the conclusion that ionization is a primary factor in determining cell permeability for acids;[13] if sufficiently great, the degree of dissociation practically controls the situation, and when chemically related acids are considered it fixes their relative penetrating power with precision; if sufficiently low, as in the fatty acid series aside from formic, some factor allied to capillary activity is dominant—though at the same time it is evident that the constitution of the other acids studied is likewise of importance in relation to the make-up of the cell surface and substance. The general result of these experiments is to indicate that while some material other than lipoids, which may be identified as protein,[14] is primarily concerned in permeability (compare Loeb, 1912[b], and Osterhout, 1913, page 141), yet the cell surface must be regarded as complex and totally misrepresented by some current simple theories of permeability, which explain fairly well the conditions of penetration for single classes of substances (compare Loeb, 1912[a], 1914, page 443: Lillie, 1913; Höber, 1914, page 349).

Comparisons with Stimulation.

Series expressing the relative activity of acids as they affect a number of processes might be compared with the data presented in this paper, were it not for the fact that in many cases such a comparison, at least on the quantitative side, would involve the possibility of a serious fallacy. This is particularly true of figures purporting to give the relative toxicity of acids, and the fallacy lies here: the times required for the same concentration of different substances to produce a given effect are commonly as-

[13] This is also indicated by the results of Dale and Mines (1911) regarding the contraction of muscle in acids.

[14] Shown also by experiments, to be described elsewhere, on the penetration of *Chromodoris* tissues by acid-salt combinations.

sumed to be proportional to the concentrations of these substances which will produce another result within some fixed time; on the contrary, the dilution-*versus*-effect curves for various substances may be of entirely different character (compare Fig. 3), and little correspondence of such acid series could reasonably be expected; moreover, the mechanism of absorption of substances from *very* dilute solutions may be quite different from that at high concentrations (compare, for example, the sense of smell in vertebrates).

(a) I wish particularly to attempt an explanation of sensory stimulation by acids, on the basis of the present data, but a more general case of stimulation may first be considered—that of induced membrane formation in the sea urchin egg (see Loeb, 1913, page 133). The order of acid effectiveness in leading to membrane formation is given, in general, as:

Monobasic fatty acids > dibasic acids > mineral acids,

which is just the reverse of that obtained in considering cell penetration. Within these groups of acids we find stated (Loeb, 1913, page 133) the following approximate order of efficiency (in 0.5 M NaCl solution).

TABLE X.

*Orders of Efficiency in Membrane Production.**

Butyric > *propionic* > *acetic* > formic.
Propionic > lactic.
Benzoic > *butyric*.
Oxalic, tartaric > *citric* > *succinic*.
HNO_3 > *HCl*(>H_2SO_4).

* The italicized instances agree with the penetration series.

Loeb (1913, page 139) comments upon the apparent disagreement of these facts with the dissociation theory, but points out that it is only the mass of acid which has actually entered the egg that is to be considered, and that the magnitude of this inwardly diffusing mass depends upon the constitution of the acid. But it will be noted that it is only in certain particulars that the membrane and penetration series agree, and that the relative position of the fatty, dibasic, and mineral acids, of formic acid, and of hydroxy substitution in the case of lactic acid, is reversed. Harvey's series for acid penetration, by agreeing fairly well with

that obtained for the *Chromodoris* tissue at the same concentration, strongly suggests the validity of my acid series for tissue cells generally, and we must conclude either that the acids leading to membrane formation act merely upon the surface of the egg, which is chemically different from the substance of the interior,[15] or else that the permeability of egg protoplasm for acids is qualitatively different from that of tissue cells. Both conclusions may be correct; but the fact of membrane formation shows that the surface of the mature egg is in its physicochemical make-up different from that of the generality of cells, and the short time in which very dilute solutions of certain acids will bring about membrane formation, as compared with their speed of diffusion through protoplasm, makes it seem certain that the parthenogenetic power of acids involves only a reaction with substances at the surface of the egg, not the egg protoplasm as a whole. This view is not incompatible with Kite's (1913) conclusion that for certain substances the permeability of egg surface and cytoplasm are the same. As far as the evidence for acids is concerned, we must regard the egg surface not as a simple thing, but as quite complex and as containing not only elements which are similar to those of tissue cells,[16] but also others or else the former in different proportions.

(b) Turning now to the matter of sense organ stimulation by acids, it may first be pointed out that the order of permeability to acids for different regions of the body of *Chromodoris* (see Table I)—*viz.*, anterior tentacles, rhinophores > gills > buccal mantle > posterior mantle edge > lateral mantle (under surface) > edge of foot > dorsal body surface—appears to be also the order of the sensitivity of these parts to acids. This statement is made on the basis of work on the behavior of *Chromodoris* by Dr. L. B. Arey,[17] and of independent experiments of my own.

Tests have been made upon marine animals, namely *Holothuria surinamensis*, *Stichopus moebii*, and *Ptychodera* sp., and upon

[15] The fact that isolated egg fragments can form membranes is no objection to this interpretation.

[16] Compare the instances in which the penetration series holds (Table X) for parthenogenesis, and the fact that cell-permeability and membrane-formation series are the same for alkalies (Harvey, 1911; Loeb, 1912[e]).

[17] I am indebted to Dr. Arey for permission to include certain of his observations in advance of publication.

earthworms, which show that, as with human taste, the limiting dilutions beyond which various acids are non-active in stimulation follow this order: fatty acids < dibasic < mineral acids, which agrees in a general way with the penetration series.

For more detailed comparison, data obtained from experiments with the worm *Eisenia foetida* may be considered. These experiments were carried out in the following way:[18]

Each worm (w, Fig. 4) was placed upon a low ridge (r–r) dividing into two compartments (a, b) a trough constructed of paraffin walls upon a ground glass plate (p); compartment a contained an acid solution, b, water once distilled, to a depth of about 1 cm., each compartment holding 50 cc. of fluid. The anterior end of the worm being immersed in the acid solution, it is stimulated by the acid, and the worm withdraws into the water. The time occupied by this response (from the instant of immersion until that of

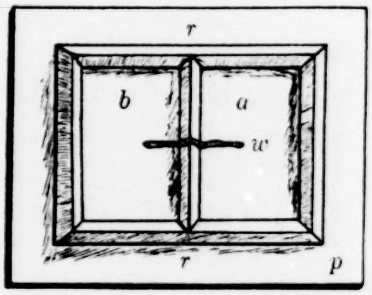

FIG. 4. View of apparatus for stimulation experiments with earthworms.

complete retraction, measured with a stop-watch) is used as an estimate of the amplitude of the reaction due to stimulation by the particular solution,—in other words, as an index of the intensity of stimulation. The figures given are the means of twenty-five tests at each concentration for each acid.

The time of response so obtained is not strictly a reaction time, as some have termed it;[19] but it is the time required for the performance of a definite act as the result of stimulation,—quite a different thing, and a quantity which we may reasonably hold

[18] Details of this method, particularly of the dozen or more recognized sources of error which surround it, and the means taken to overcome and estimate these complicating factors—such as the size of the animals, their previous history, the proportion of their length immersed, etc.—cannot be described here. Posterior stimulation was also studied.

[19] Parker and Metcalf (1906), Hurwitz (1910), Shohl (1914).

to be proportional to stimulus intensity.[20] I mean by a stimulus the alteration produced in a receptor,[21] not the concentration (density) of the stimulating agent.

In comparing these curves of response (Fig. 5) to several dilutions of a number of acids, the following are the outstanding

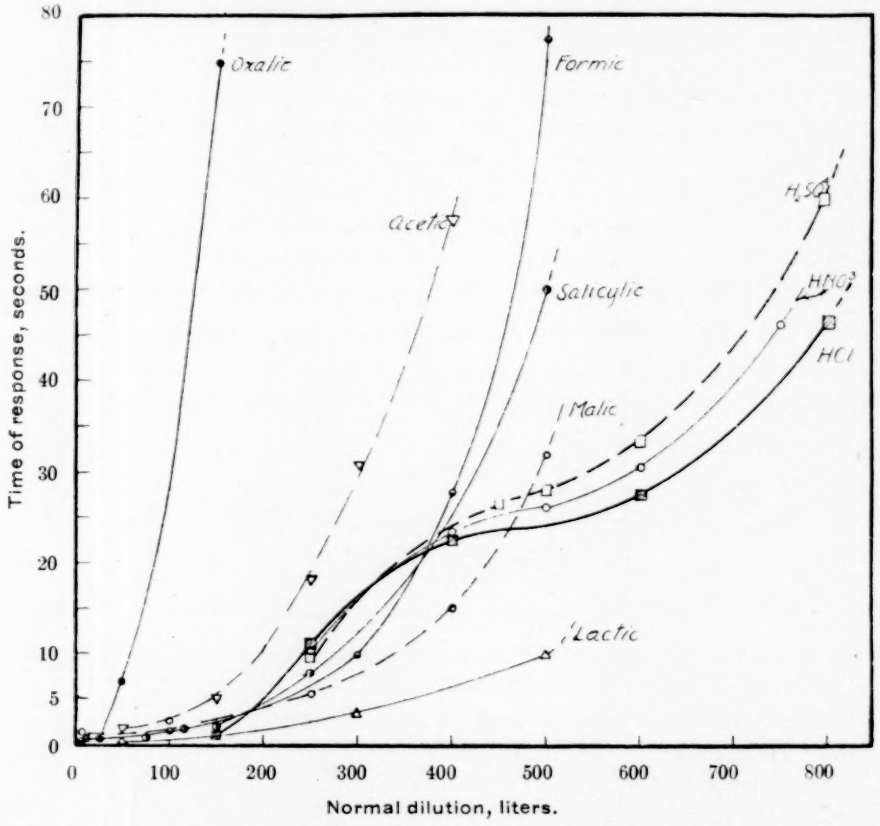

FIG. 5. Time of response to dilutions of nine acids, for the earthworm (see text). No measurable responses were obtained above the dilutions shown.

points and the only ones which will be considered here: (1) Different types of curves are obtained (a) with strong mineral acids,

[20] Or to the difference between this intensity and a factor expressing the tendency of the worms to progress normally in a forward direction. There are further complexities when the stimulation of the tail end is considered.

[21] In the present case, rather the integral of the partial stimulations experienced by the individual ceptors, according to whatever scheme this integration is accomplished in the nervous system of the animal. Except for the prostomium, there is good morphological ground for the opinion that this scheme may be fairly simple.

and (b) with weak organic acids.[22] (2) The strong acids follow the order of their ionization, but this at such high dilutions that the correspondence probably has little real reference to dissociation. (3) The weak acids fail to stimulate at lower dilutions than do the strong acids. (4) The weak acids are more stimulating in proportion to their ionization than would be predicted if this were the only factor involved.

The points numbered (1), (3), and (4) find their exact counterparts in the penetration series, but the time quantities involved make it obvious that the ceptor surface only is concerned in stimulation. That there is no detailed correspondence in the position of acids in the two series is not surprising, since this worm lives commonly in manure piles, and its chemoreceptors are therefore probably specialized in a peculiar way.

Points (3) and (4) have, of course, long been recognized in taste stimulation (human), and indeed appear universally when chemoreceptors are considered. Various explanations have been suggested to account for these facts, such as the possible greater ionization of the part of the acid at the cell surface, etc.; but it seems to me that explanations of this type cannot hold. The parallelisms between the stimulation series and the penetration series show unmistakably that chemical stimulation involves the solution of the stimulant in the surface of the ceptor cell; and from such solutions or combinations the requisite electrochemical effects can readily be derived (compare Beutner, 1912).

Further evidence in this direction is afforded by the fact that, if the worms are successively stimulated at short intervals (30 seconds), data are obtained such as are shown in Fig. 6. Two aspects of these curves are significant: (1) the temporary lengthening of the time of response after several stimulations,[23] which may be related to the fact that acids tend to produce at first a decrease in permeability (Osterhout, 1914); and (2) the fact that after a certain period the time of response begins to lengthen very rapidly. Now, the speed with which this falling off occurs varies with the different acids, and was found (at 0.02 N to 0.01 N) to follow the order:

Salicylic, oxalic > HCl, HNO_3, H_2SO_4 >
formic > lactic > malic > acetic.

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[22] Oxalic acid was found to be so toxic that its curve cannot be considered with the rest.

[23] Frequently preceded by a heightened irritability.

The agreement of this order with that of cell penetration at these concentrations (Table VII) is striking, and indicates that as the ceptor is entered by acids, its irritability is immediately decreased.[24]

From this point of view it is comprehensible that in secondary sense cells, as of the vertebrate taste bud, we find nerve terminals in connection with the surface of the ceptor, not penetrating its interior.

We must regard this ceptor surface as a complex structure, and thus far the indication is that it contains both fatty substances and proteins.[25]

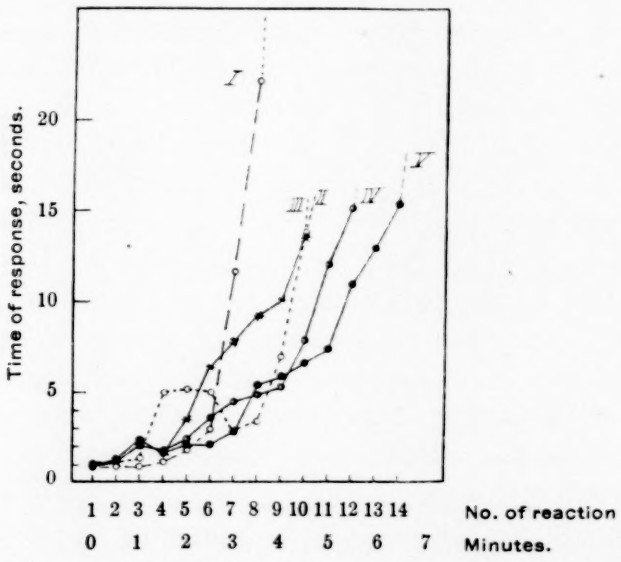

FIG. 6. Effect of successive stimulation on the time of response to 0.133 N salicylic acid; individual worms I to V.

SUMMARY.

1. The method employed for the study of acid penetration depended upon the selection of pieces of tissue from the edge of the mantle of the nudibranch *Chromodoris zebra* Heilprin; these pieces give reasonably uniform and reproducible results when the following conditions are observed: (a) the animals selected are of the same size and of the same kind and degree of coloration; (b) the

[24] The responses of the earthworm depend upon many scattered chemoreceptors, which are naturally not all influenced at exactly the same time.

[25] Compare Loeb (1912[b]), Osterhout (1913), and Loeb and Beutner (1913).

pieces of tissue are taken from corresponding locations, are treated in the same way, and submitted to the action of acids at a constant temperature. This tissue contains an intracellular blue substance, which is turned pink at about $p_H = 5.6$. The time required for this change when immersed in acid solutions is measured under conditions favorable to judgment of the precise moment of color change.

2. The relative penetrating speed of eighteen acids, measured over a range of dilutions, shows that two sets of factors are concerned in penetration. One of these is ionization, the other varies with the constitution of the acid. It is indicated that the cell surface is complex, and contains fatty substances, proteins, and probably other materials.

3. Comparison of these data with observations on artificial parthenogenesis (Loeb) suggests that the stimulus to membrane formation in the sea urchin egg depends upon the union of the acid with the surface of the egg, which is of quite different proportional composition from the cytoplasm of tissue cells.

4. Measurements of the stimulating power of various acids show, in the light of the penetration series, that the surface of chemoreceptors is of complex constitution, probably containing proteins and fatty substances; they show also that the process of stimulation involves the union of the stimulating agent with some constituent of the ceptor surface.

AGAR'S ISLAND, BERMUDA.

The following note was added while this paper was in press. In Harvey's later paper (*Z. physik.-chem. Biol.*, 1914, i, 463–478), which was until a few days ago unknown to me, it is shown that the dilution-*versus*-penetration curves for butyric and hydrochloric acids cut across each other very much as found for strong and weak acids with the tissue of *Chromodoris*. This agreement is rather striking and adds materially to the strength of the interpretation put upon these facts. With the testis epithelium of *Stichopus*, as with the integument of *Chromodoris*, the strong acid penetrates more easily from dilute solutions than does the fatty acid. The same condition appears in Harvey's experiments upon luminous bacteria (*Biol. Bull.*, 1915, xxix, 309). I must therefore disagree with his view that "living cells behave as if they were droplets of oil or fat solvents" (Harvey, 1914, *Z. physik.-chem. Biol.*, i, 476).

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THE RATE OF OXIDATIONS IN REVERSED ARTIFICIAL PARTHENOGENESIS.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, February 1, 1916.)

In 1913 Loeb[1] showed that by suitable treatment unfertilized eggs of the sea urchin *Arbacia*, in which development had been artificially initiated, could be caused to revert to their original inactive condition. This was accomplished by placing the eggs in solutions of sodium cyanide or chloral hydrate immediately after a preliminary treatment with butyric acid or an alkali or with alkaline hypertonic solution.

If the treatment with butyric acid or alkali only was applied, or if, after this treatment, the eggs were placed in the sodium cyanide or chloral hydrate solutions for an insufficient length of time, they would disintegrate on transference to normal sea water. On the other hand, eggs which had received a sufficiently long treatment with sodium cyanide or chloral hydrate, on transference to normal sea water, remained intact and could be either fertilized or, in some cases, induced to develop again by the usual methods of artificial parthenogenesis. Clearly, a reversal of the effects induced by the alkali or acid treatment had taken place.

The chloral hydrate and sodium cyanide treatments, as Loeb pointed out, cause the reversal of the induction of development by suppressing the developmental changes due to the alkali or acid treatment.

Until more is known as to the nature of the changes taking place in the egg on membrane formation, it is difficult to form any idea as to the nature of the reaction whose rate is decreased, but in view of the marked changes in the rate of oxidations in the egg coincident with fertilization or artificial membrane production, it seemed of interest to follow the course of the oxidations during the process of reversion described by Loeb.

[1] Loeb, J., *Arch. Entwcklngsmechn. Organ.*, 1914, xxxviii, 277.

In the following experiments the rate of oxidations was measured at different stages in the method used by Loeb,[2] and the results show clearly that after the reversal which he described the oxidations indeed continue at the normal rate existing in the unfertilized, untreated egg.

I. Method.

The method used in the determination of the rate of oxygen consumption was that of Siebeck.[3] This method possesses the great advantage that only relatively small amounts of egg suspension are required for a determination, and also that the rate can be ascertained at any desired moment during a determination. The readings are made by means of a water manometer, and the size of the apparatus is so arranged that 1 mm. pressure on the water manometer corresponds almost exactly to an oxygen consumption of 1 c. mm.

Heilbrunn[4] has recently objected that results by this method are not reliable, in that substances diffusing out of the egg into the solution cause a lowering of its solubility for oxygen, thus yielding a low result. Apart from the fact that an absolute value for the consumption of oxygen is neither necessary nor desired in such experiments, this source of error, though it undoubtedly exists, is shown to be negligible by means of simple controls suggested by the author of the method and readily carried out with *Arbacia* eggs.

In order to give some idea of the method of manipulation, a typical experiment will be briefly described.

A suspension of *Arbacia* eggs, after thorough mixing, was divided into six equal parts. Two of these parts were used for the control determination of oxygen consumption by the unfertilized eggs. The controls having been set aside, the remaining four portions were mixed, treated for $2\frac{1}{2}$ minutes with butyric acid (2.0 cc. 0.1 N acid to 50 cc. sea water), and immediately transferred to 50 cc. sea water + 0.4 cc. 0.1 per cent sodium cyanide

[2] Loeb, *Science*, 1913, xxxviii, 749; *Arch. Entwcklngsmechn. Organ.*, 1914, xxxviii, 277.

[3] Siebeck, R., Abderhalden's *Handb. biochem. Arbeitsmethoden*, 1915, viii, 33.

[4] Heilbrunn, L. V., *Science*, 1915, xlii, 615.

solution. This suspension was then divided into two equal portions, each one-third of the original amount of eggs taken. The first of these was washed four times in the sea water + sodium cyanide solution and finally divided into two equal portions, each one-sixth of the original amount, and placed in stoppered flasks. The other portion which had been treated with butyric acid was washed four times in normal sea water, and finally it too was divided into two equal portions. As a result of this manipulation, there were obtained six equal suspensions of eggs; two of these, the controls, had received no treatment, two had been treated with butyric acid and subsequently kept in the sea water cyanide mixture, while two had been treated with butyric acid and were subsequently kept in normal sea water in which they disintegrated in the course of a few hours.

Each portion received, as nearly as possible, an equal amount of washing, and, in order to equalize any error due to loss of eggs in the process, even the untreated eggs were subjected to the same washing, so that the rates of oxidation in the six final samples are fairly closely comparable. In order to reduce the volume of the suspension to the amount required for the oxygen determination, *viz.*, 2.5 cc., an attempt was made at first to hasten the process by concentration in a slow speed centrifuge. It was found, however, that even when the greatest care was exercised, the eggs sometimes suffered through this treatment and the expedient was resorted to of allowing the eggs to settle by gravity in a centrifuge tube and decanting the supernatant sea water until the desired volume was obtained. The suspension was then transferred, by means of a wide-nosed pipette, to the flask in which the determination was carried out.

The determinations of oxygen consumption were in all cases carried out at a temperature of 25°C., kept constant to within 0.02°C. No matter what previous treatment the eggs had received, the final measurements were made in sea water in every case.

In all the experiments described in this paper, a similar method of manipulation was used.

II. Oxidations in Reversal.

The rates of oxidation in eggs treated with butyric acid and kept subsequently in sodium cyanide sea water are given in Table I. On removal from the sodium cyanide solutions, the eggs were, of course, washed and allowed to remain in normal sea water for about 1 hour before proceeding with the oxygen determination.

TABLE I

No. of experiment	1	2	3	4	5	6	7	8	9	10
	Oxygen consumed, c. mm. per hr.									
Untreated eggs	27	25	30	30	18	28	20	24	10	17
Eggs treated with butyric acid	75	71	62	53	50	49	27	80	17	56
Eggs treated with butyric acid after about 19 hours in sea water + NaCN	53	40	52	29	21	37	21	22	10	17

Table I shows that, with the exception of the first three experiments in which the technique had not been so well developed and also in Experiment 6, the rate of oxidations after about 19 hours in the sodium cyanide solution, is nearly the same as for the untreated eggs.

The subsequent behavior of the eggs was followed for at least 1 day further. It was noted (1) whether the eggs remained normal on removal from the sodium cyanide solution, which was nearly always the case; (2) whether they could be fertilized, this being nearly always possible, a considerable proportion developing to swimming larvæ; and (3) whether they could be made to develop by repeating the treatment with butyric acid followed by hypertonic sea water, as in the ordinary method of artificial parthenogenesis. In some experiments a large percentage could be caused to develop to swimming larvæ by such treatment indicating a fairly complete reversal of the effects of the first butyric acid treatment, though in other experiments the reversal tested by this means proved less satisfactory. In such cases, however, this result found its explanation in the figure obtained for oxygen consumption, the reversion of the latter not being complete. The completeness of the reversal was also tested by simple treatment

of the eggs for about 20 minutes with hypertonic sea water or removal from the sodium cyanide solution. If the eggs could be caused to develop by such treatment it was indicated that the effect of the original butyric acid treatment still persisted and that therefore the reversal was not complete. In two instances a large percentage of the eggs—about 15 per cent—underwent segmentation and developed to swimming larvæ on treatment in this manner. In the remaining experiments none or very few eggs responded to such treatment.

In some of the experiments the eggs which had remained about 19 hours in the sea water and sodium cyanide mixture were subjected again to the butyric acid treatment and their rate of oxidations was determined. Table II gives the result of these determinations.

TABLE II.

No. of experiment	6	7	8	9	10
	Oxygen consumed, c. mm. per hr.				
Untreated eggs	28	20	24	10	17
Eggs treated with butyric acid	49	27	80	17	56
Eggs treated with butyric acid after 19 hours in sea water + NaCN	37	21	22	10	17
Eggs treated with butyric acid after 19 hours in sea water + NaCN, but treated again with butyric acid	31	49	31	19	29

In all cases save in Experiment 6, the rate of oxidations rose again on retreatment with butyric acid, though the original rate was reached only in two instances. These results are further confirmation of the reversal of the effect induced by the first butyric acid treatment.

The rate of oxidations in eggs treated with ammonium hydroxide and kept subsequently in sodium cyanide sea water was also determined. These results are given in Table III. The method of manipulation was similar to that already described for the butyric acid treatment. The eggs remained for 20 minutes in a mixture consisting of 0.3 cc. 0.1 N ammonium hydroxide with 50 cc. sea water, being then transferred directly to the sodium cyanide solution.

TABLE III.

No. of experiment	11	12	13	14	15	16
	Oxygen consumed, c. mm. per hr.					
Untreated eggs	19	18	24	11	16	9
Eggs treated with NH_4OH	26	28	37	49	48	31
Eggs treated with NH_4OH after about 19 hours in sea water + NaCN	28	38	35	15	17	11
Eggs treated with NH_4OH after about 19 hours in sea water + NaCN and treated again with NH_4OH	53	47	61	29	42	21

In the last three experiments the technique employed was better than in the three earlier ones. These results are substantially the same as those obtained in the experiments with the butyric acid treatment. The rate of oxidations, on removal from the cyanide solution, was approximately the original rate and increased again on retreatment with ammonium hydroxide.

After treatment with the alkali and transference to sea water the eggs disintegrated, though after about 19 hours in the sodium cyanide solution, on transference to normal sea water, they remained intact and normal. Such eggs on removal from the cyanide could be fertilized by sperm, and also in most cases caused to develop on retreatment with ammonium hydroxide or butyric acid and hypertonic sea water. When these eggs were placed directly into hypertonic sea water for about 20 minutes, after removal from the sodium cyanide solution, a small percentage segmented and a few even developed to the blastula stage, indicating that some effects of the original alkali treatment still remained and that reversal was not quite complete.

III. Fate of Eggs Which Did Not Receive the Cyanide Treatment.

The rates of oxidations in eggs which had received the acid or alkali treatment and had not been transferred to the cyanide solution but kept instead, until next day, in normal sea water, were also determined and the results are shown in Table IV.

TABLE IV.

No. of experiment.....	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	Oxygen consumed, c. mm. per hr.													
Untreated eggs.....	30	30	18	28	20	24	10	17	19	18	24	11	16	9
Treated eggs (acid or alkali treatment) about 1–2 hours after treatment.....	62	53	50	49	27	80	17	56	26	28	37	49	48	31
Treated eggs next day.	39	58	62	31	34	38	20	15	52	80	93	23	53	19

It will be noted that the figures for the oxidations on the second day are somewhat inconsistent. In some experiments the rate of oxidations has fallen to a figure not much greater than the original rate, while in other experiments it remains as high as or even higher than the increased rate due to membrane formation by acid or alkali treatment.

There is also considerable variation exhibited in the magnitude of the response to the acid or alkali treatment, in some experiments an increase in oxidations of about 400 per cent is noted, while in some the increase is less than 100 per cent.

The explanation for some of these apparent inconsistencies is fairly simple and lies in the fact that the magnitude of the response of sea urchin eggs to a given treatment by acid or alkali is not always the same, the variations being due to differences in sensitivity in the eggs of individual females and perhaps also to differences in room temperature during the several experiments. This accounts for the varying percentage increase in the rate of oxidations on a given treatment with acid or alkali and also for an unequal resistance to the disintegrating effects of such treatment, observed in the eggs of different females. It also accounts for differences observed in the rate of oxidations in untreated eggs preserved for some time in sea water in the laboratory. These frequently show a considerable spontaneous increase in a relatively short time, as will be seen from the figures given in Table V.

TABLE V.

No. of experiment	8	9	10	11	12	13	14	15	16
	Oxygen consumed, c. mm. per hr.								
Untreated eggs	24	10	17	19	18	24	11	16	9
Untreated eggs kept over night in normal sea water	29	17	17	67	36	80	23	26	16

In all these experiments except No. 10 a considerable increase has taken place. The magnitude of this increase, however shows considerable variation.

IV. The Spontaneous Increase in Oxidations in Unfertilized Eggs.

In order to account more fully for the apparent inconsistency of the results in Tables IV and V, it is necessary to consider in some detail the spontaneous increase in oxidations in the eggs on standing. An endeavor was made to ascertain what factor is responsible for the phenomenon.

Loeb[5] showed that some of the eggs of certain females of the sea urchin *Strongylocentrotus purpuratus* formed fine gelatinous membranes spontaneously, when allowed to remain for some time, about 24 to 48 hours, in sea water at relatively low temperature. He found that these eggs sometimes segment, and if kept at a low temperature may even reach the sixteen cell stage. Loeb also found that if eggs which form membranes spontaneously are treated with hypertonic sea water, as in the usual method of artificial parthenogenesis, some will develop to larvæ.

In the eggs of *Arbacia* such spontaneous membrane formation is hardly ever observed. This may be due to the fact that in *Arbacia* eggs the membrane lies much more closely on the surface of the egg than is the case in *Strongylocentrotus*, and is therefore more difficult to observe. It is certainly true of the fertilization membrane and even more true of the membrane formed after the butyric acid treatment. A spontaneous segmentation is sometimes noted in *Arbacia* eggs, but this never goes beyond the two or three cell stage. It is possible, therefore, that spontaneous membrane formation or some spontaneous alteration of

[5] Loeb, *Arch. Entwcklngsmechn. Organ.*, 1913. xxxvi, 626.

the cortical layer also occurs, in the eggs of some females, in *Arbacia*.

Artificial membrane formation in *Strongylocentrotus* is always accompanied by a considerable increase in oxidations, whether the membranes have been called forth by butyric acid or by cytolytic agents such as saponin.[6] While the point has not been tested, it is reasonable to suppose that spontaneous membrane formation is also accompanied by a considerable increase in oxidations.

Loeb suggested that the spontaneous membrane formation in *Strongylocentrotus purpuratus* might be caused by the alkalinity of the sea water and it was thought desirable to ascertain whether the spontaneous increase in oxidations in *Arbacia* eggs takes place more readily in a slightly alkaline than in a slightly acid sea water. To this end the following simple experiment was carried out. Eggs in equal quantities were placed in a number of finger bowls. One-half of these bowls contained an artificial sea water made by mixing 100 cc. 0.5 M NaCl, 2.2 cc. 0.5 M KCl, 1.5 cc. 3/8 M $CaCl_2$, 7.5 cc. 3/8 M $MgCl_2$, and 3.5 cc. 3/8 M $MgSO_4$. This solution is acid to neutral red, while the normal sea water is slightly alkaline. All the various lots of eggs received an equal amount of washing. At intervals the rates of oxidations in eggs taken from the normal and from the artificial sea water were compared, the determination in both cases being performed in normal sea water. For this purpose the eggs from the artificial sea water were washed in normal sea water immediately before making the determination. The results are contained in Table VI.

[6] Loeb, J., and Wasteneys, H., *J. Biol. Chem.*, 1913, xiv, 469.

TABLE VI.

Length of time eggs had remained in solution.	Normal sea water (slightly alkaline).		Artificial sea water (slightly acid).	
	Oxygen consumed per hr.	Condition of eggs.	Oxygen consumed per hr.	Condition of eggs.
hrs.	*c. mm.*		*c. mm.*	
3	12	Normal.	10	Normal.
20	19	Majority normal, some disintegrated.	10	"
26	19	Majority normal, some disintegrated.	13	"
44	57	Agglutinated and disintegrated.	27	Majority normal, some cytolyzed.
$1\frac{1}{2}$	11	Normal.	9	Normal.
19	11	"	10	"
44	73	All disintegrating.	19	Majority normal, some disintegrating.
1	10		9	
$5\frac{1}{2}$	19		18	
$8\frac{1}{2}$	17		16	
24	34	Many disintegrating.	37	
47	49	All "	29	Majority normal.
19	8		9	
29	31	Some disintegrating.	16	Normal.

These results, while incomplete, indicate that alkalinity plays an important rôle in the spontaneous increase in oxidations in the sea urchin egg, and though more experiments are required to determine its degree of responsibility, it is evident that the spontaneous disintegration of the eggs in sea water can be strongly inhibited by reducing the alkalinity.

The rôle played by the alkalinity of the sea water is also strikingly illustrated in experiments with butyric acid treatment of the eggs. In these experiments the butyric acid solution was made with artificial sea water possessing a faintly acid reaction instead of ordinary sea water which is faintly alkaline. The eggs were transferred from the butyric acid solution directly to arti-

ficial sea water or to normal sea water as the case might be, and the relative increase in the rate of oxidations was determined 1 hour later. The results are given in Table VII.

TABLE VII.

Treatment.	2½ min. in 1 cc. 0.1 N butyric acid to 50 cc. artificial sea water.	5 min. in 1 cc. 0.1 N butyric acid to 50 cc. artificial sea water.	5 min. in 1 cc. 0.1 N butyric acid to 50 cc. artificial sea water.	3 min. in 2 cc. 0.1 N butyric acid to 50 cc. artificial sea water.
Untreated eggs.....	21.5	11	7	11
Treated eggs transferred to normal sea water.....	26.0	18	16	46
Treated eggs transferred to artificial sea water.....	16.0	9	6	11

When the eggs are transferred from the butyric acid solution to the faintly acid artificial sea water, the membranes are not formed so well as when the eggs are transferred to the slightly alkaline sea water. There is also some loss in the treatment with butyric acid in artificial sea water as the eggs in such a solution have a strong tendency to stick to the sides of the vessel. This accounts for the decrease in the rate of oxidations shown by the treated eggs when transferred to artificial sea water.

In the slightly acid sea water no increase in the rate of oxidations takes place following butyric acid treatment. The increase on transference to normal sea water shows, however, that the butyric acid treatment was sufficient for the purpose. The presence of excess of hydroxyl ions seems, therefore, to be necessary for the activation of the processes resulting in increased oxidations.

This result was forecasted by Loeb[7] who found that eggs of *Strongylocentrotus purpuratus* when treated with butyric acid dissolved in neutral 0.5 M Ringer solution and transferred to neutral 0.5 M Ringer *per se*, formed only abnormal membranes and neither disintegrated nor developed. He suggested that the butyric acid treatment had not brought about the usual increase in oxidations but was inclined to attribute the abnormal behavior to causes other than hydroxyl ion concentration.

[7] Loeb, *Biol. Bull.*, 1915, xxix, 103.

One other possibility suggested itself in connection with the spontaneous increase in oxidations. It was observed that, when a considerable quantity of eggs remains in contact with only a small amount of sea water, a few of the eggs occasionally form membranes. It was also noticed that under such conditions the eggs appeared to disintegrate earlier than eggs which are less closely packed. This might be due to the presence, in considerable amount, in the surrounding sea water, of substances exuded by the eggs, which induced that critical change in the cortical layer of the egg responsible for membrane formation. Such eggs should exhibit an increased rate of oxidation.

In order to test this point, a suspension of eggs was equally divided. One portion was placed in a small amount of sea water under conditions of minimal evaporation while the other equal portion was placed in a relatively much larger quantity of sea water which was frequently renewed. Determinations of the rate of oxidations were then made at intervals, with the result given in Table VIII.

TABLE VIII.

No of experiment.....	1			2			
No. of hours under given condition.	4	23	26	4½	9	22	27½
	Oxygen consumed, c. mm. per hr.						
Eggs kept in a small quantity of sea water.....	23	73	71	9	10	21	32
Eggs kept in a large quantity of sea water which was frequently renewed.	22	38	52	10	24	25	48

It is evident from these figures that the spontaneous increase in the rate of oxidations continues in spite of dilution and removal of the egg exudates and is not even markedly inhibited thereby.

As a further control on the behavior of eggs on standing in sea water, a few determinations were made of the rate of oxidations in normal untreated eggs which had been kept over night in the sea water and sodium cyanide mixture. It is hardly necessary to state that the eggs were well washed in sea water and allowed to stand for some time after removal from the cyanide solution

before proceeding with the determination. These results are given in Table IX.

TABLE IX.

No. of experiment	14	15	16
	Oxygen consumed, c. mm. per hr.		
Untreated eggs	11	16	9
Untreated eggs on following day	23	26	16
Untreated eggs kept over night in NaCN solution	12	19	11

The changes responsible for the spontaneous increase in oxidations in unfertilized eggs on standing are evidently inhibited by preserving them in the sodium cyanide solution. This agrees with the old observation of Loeb and Lewis[8] that in potassium cyanide the life of unfertilized sea urchin eggs is prolonged.

On subjecting these eggs which had remained over night in the cyanide solution to treatment for 20 minutes with 0.3 cc. 0.1 N ammonium hydroxide in 50 cc. sea water, the oxidations were increased to an amount approximating that reached by eggs from the same lot when treated on the previous day, thus proving that the cyanide treatment had kept the eggs intact. This is shown by the results given in Table X.

TABLE X.

No. of experiment	14	15	16
	Oxygen consumed, c. mm. per hr.		
Untreated eggs	11	16	9
Eggs after NH_4OH treatment	49	48	31
Untreated eggs kept over night in NaCN solution	12	19	11
Eggs kept over night in NaCN solution and then treated with NH_4OH	34	42	39

In some of the experiments described, it was thought desirable to follow further the fate of eggs which had received the butyric

[8] Loeb, J., and Lewis, W. H., *Am. J. Physiol.*, 1901-02, vi, 305.

acid treatment. Accordingly, the treated eggs were preserved until the following day and the rate of oxidations was once more determined. A comparison of the figures obtained for treated eggs with those for untreated eggs kept during a similar period is of interest, especially in connection with the question of spontaneous oxidations. These results are given in Table XI.

TABLE XI.

No. of experiment.	Time after treatment.	Oxygen consumed per hr.		Condition of treated eggs on second day.
		Untreated.	Treated.	
		c. mm.	*c. mm.*	
8	1 hr.	24	80	
	Next day.	29	38	All disintegrated.
9	1 hr.	10	17	
	Next day.	17	20	" "
10	1 hr.	17	56	
	Next day.	17	15	" "
11	1 hr.	19	26	
	Next day.	67	52	Some intact.
12	1 hr.	18	28	
	Next day.	36	80	Majority intact.
13	1 hr.	24	37	
	Next day.	80	93	70 per cent intact.
14	1 hr.	11	49	
	Next day.	23	23	Majority disintegrated.
15	1 hr.	16	48	
	Next day.	26	53	" "
16	1 hr.	9	31	
	Next day.	16	19	" "

The untreated eggs were nearly always intact on the second day. It will be noticed that in some experiments the treated eggs show a diminution in oxidations on further standing, while in other experiments an increase occurs.

These results, at first glance somewhat anomalous, may be explained as follows: If the idea, that the spontaneous increase in oxidations is due to the changes in the cortical layer which underlies membrane formation, is correct, then it is also probable that such alterations in the cortical layer of the egg will continue, influenced by the excess of OH ions in the sea water, even after membrane formation has been induced by acid treatment. In such a case the rate of oxidations will also continue to increase. Thus, in Experiments 11 and 13, the oxidations were not greatly raised by the butyric acid treatment. It happened, however, that in the untreated eggs the oxidations increased spontaneously to a high figure; consequently, in the treated eggs, the oxidations continued to increase, also spontaneously, until a correspondingly high figure was attained. In the other experiments, with the exception of No. 12, the oxidations increased considerably on treatment. Such eggs show no or only very slight further increase in oxidations on the following day, the maximum having already been reached as a result of the acid treatment. Generally, owing probably to disintegration, the oxidations have again diminished on the next day, by an amount roughly proportional to the degree of disintegration.

Another explanation is also possible though perhaps less probable, and is as follows: Warburg and Meyerhof[9] found that the oxidations in unfertilized sea urchin eggs are only slightly less after disintegration than before. Warburg[10] found that when fertilized eggs are caused to disintegrate, the rate of oxidations returns to the value for an equal quantity of unfertilized eggs. He concluded that the increase in oxidations on fertilization is bound up in the existence of the fertilization membrane. This idea resembles somewhat the *Hauptathmung* and *akzessorische Athmung* of Battelli and Stern.[11] Warburg[12] showed that the membrane called forth by the butyric acid treatment acts like the fertilization membrane in increasing the rate of oxidations. Assuming that on disintegration the rate of oxidations in the treated eggs similarly falls again to the rate in the unfertilized eggs, which

[9] Warburg, O., and Meyerhof, O., *Arch. ges. Physiol.*, 1912, cxlviii, 295.
[10] Warburg, O., *Arch. ges. Physiol.*, 1914, clviii, 189.
[11] Battelli, F., and Stern, L., *Biochem. Z.*, 1914, lxvii, 443.
[12] Warburg, *Z. physiol. Chem.*, 1910, lxvi, 305.

is probable, and assuming that the spontaneous increase in oxidations in unfertilized eggs is not caused by spontaneous membrane formation but by the action of the OH ions of the sea water independently of membrane formation, the results in Table XI can be readily explained.

In Experiments 12 and 13 the oxidations are slightly raised by the acid treatment and the spontaneous increase in the untreated eggs next day is considerable. A spontaneous increase has similarly occurred in the treated eggs, and as the eggs had not disintegrated the figures remained high. In the remaining experiments in which the treated eggs had disintegrated on the second day, the increased rate of oxidations has diminished again to a value corresponding to that for the untreated eggs. Where no spontaneous increase has taken place, the increased rate due to treatment has dropped to the original value. In those cases where some spontaneous increase took place the final value on the second day for both treated and untreated eggs agrees closely.

V. What Causes the Reversal Effects?

It has been shown that after the reversal of the action of butyric acid as demonstrated by Loeb, the rate of oxidations is the same as the rate existing before treatment, and the several questions subsidiary to the main issue have also been answered fairly satisfactorily.

The next question for consideration would appear to be, How does the sodium cyanide solution accomplish the reversion? We know that it suppresses oxidations and development, but there is no *a priori* reason why a temporary suspension of induced cell activities should result in a reversion to the reactions of an unfertilized egg. It is to be remembered, in this connection, that, as Loeb showed, chloral hydrate acts similarly to sodium cyanide in causing a reversion, and the chloral hydrate compared to sodium cyanide has a relatively slight effect in depressing the oxidations in sea urchin eggs. The following experiments carried out with *Arbacia* eggs serve to illustrate this.

TABLE XII.

	Relative oxygen consumption.
Fertilized eggs in normal sea water	1.00
" " " 50 cc. " " + 0.22 cc. 0.1 per cent NaCN	0.15
" " " 50 " " " " 0.22 " 0.1 " " "	0.27
" " " 50 " " " " 0.15 " 0.1 " " "	0.20
" " " 40 " " " "10.00 " 0.5 " " chloral hydrate	0.67
Fertilized eggs in 42.5 cc. sea water + 7.5 cc. 0.5 per cent chloral hydrate	0.61
Fertilized eggs in 44.0 cc. sea water + 6.0 cc. 0.5 per cent chloral hydrate	0.73

These experiments were made in the course of another investigation and the concentrations are not those most suitable for the reversion of the effects induced by butyric acid. The concentrations used in the reversal experiment are 0.4 cc. 0.1 per cent sodium cyanide or 5 cc. 0.5 per cent chloral hydrate per 50 cc. sea water mixture, consequently the depression of oxidations during the reversal process is probably less for chloral hydrate and greater for sodium cyanide than is shown by the above figures.

It is probable, therefore, that the main factor concerned in bringing about reversion is the suppression of the developmental processes, the lowering of the rate of oxidations being merely the means of suppressing cell division.

In conclusion, the writer wishes to express his thanks to Professor Jacques Loeb for advice and criticism.

SUMMARY.

1. The rate of oxidations was determined in sea urchin eggs in which development had been initiated and later reversed by the methods of Loeb.

2. It was found that the rate of oxidations, which increases after initiation of development, returns, after reversal, to approximately the original rate of the untreated eggs.

3. It was found that on retreating the reversed eggs so as to initiate development once more the oxidations are again increased.

4. Eggs in which initiated development had been reversed could be fertilized by sperm, and also in most cases could be caused to develop by the usual methods of artificial parthenogenesis.

5. The oxidations in eggs in which development had been initiated but not reversed were studied, as were also the oxidations in untreated eggs from the same batch.

6. It was observed that the rate of oxidations in untreated eggs rose spontaneously on standing, sometimes showing a 300 per cent increase.

7. The probable cause of the spontaneous increase was found in the alkalinity of the sea water.

8. It was shown that the oxidations do not increase in eggs treated with butyric acid when transferred to faintly acid sea water.

9. The cause of the reversal is discussed, and it is shown to lie mainly in the suppression of developmental processes.

UNITS OF REFERENCE FOR BASAL METABOLISM AND THEIR INTERRELATIONS.

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The data upon which this paper is based were collected in connection with the general Use of Food Experiment which has been conducted for the past 8 years at the Agricultural Experiment Station of the University of Missouri. The animals studied were of the Hereford-shorthorn cross of beef cattle. In addition to extensive feeding and slaughtering trials and chemical analyses, maintenance and digestion trials were run and measurements were made of the surface area, blood weight, body weight, empty body weight, fat-free empty weight, and total protoplasmic tissue. The total nitrogen by analysis was used as a measure of the total protoplasmic tissue.

The data have already been given[1] in some detail and it has been pointed out that the condition of the cattle influences the relations between body weight and empty weight, and between empty weight and weight of nitrogen, surface area, and blood weight and volume. The relations are, however, constant for like conditions. It is the purpose here to discuss part of the data there presented together with a few additional data. It is hoped that some light will be thrown upon the general question of what is the best unit of reference for basal metabolism and what are the relations, if any, between these units. The latter part of the question will be considered first.

[1] Trowbridge, P. F., Moulton, C. R., and Haigh, L. D., The Maintenance Requirement of Cattle, *Missouri Agricultural Experiment Station, Research Bulletin 18*, 1915.

I.

Perhaps the simplest conception of the needs of animals for food is based upon the generally observed fact that larger animals need more food than smaller animals and that the needs are roughly proportional to the mass, being about the same for each unit of mass for like animals under like conditions. The active part of this unit of mass may be a large or a small part of the unit in the case of beef cattle, as the author has shown elsewhere.[1]

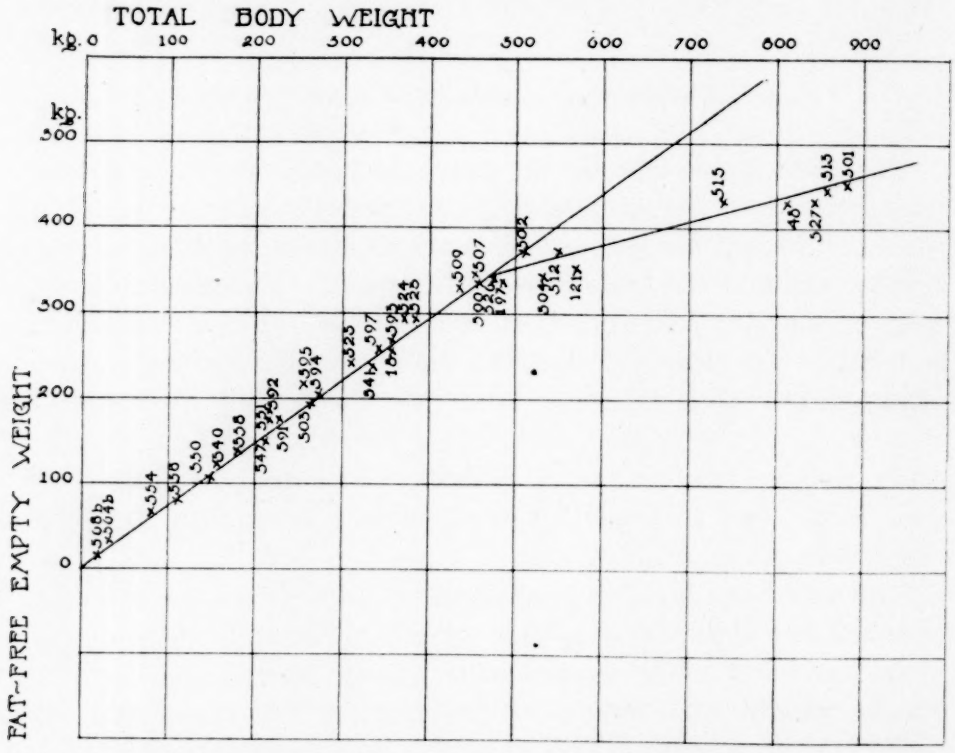

FIG. 1. Relation between fat-free empty weight and total body weight of 35 beef cattle.

The amount of the nitrogenous, or protoplasmic, tissue is a measure of the active machine within the animal. The percentage of this tissue may be 50 per cent more in one beef steer than in another, depending upon the relative fatness of the animals.

Fig. 1 shows the relation between total body weight and fat-free empty weight of 35 beef steers. The animals range in age from calves at birth to 4 and 5 year olds, and in weight from 17.7 to 883.5 kg. The condition, or fatness, varied from emaciation

to extreme fatness. In spite of these variations a fairly constant ratio is shown for the animals of poor to good condition. Only the prime and overdone steers show a greatly decreased proportion of fat-free weight. For the purpose of the general experiment most of the animals had been divided into three groups. Group I was full fed from birth, Group II was fed for maximum growth without the laying on of appreciable fat, and Group III was fed for retarded growth or to represent poor farm conditions.

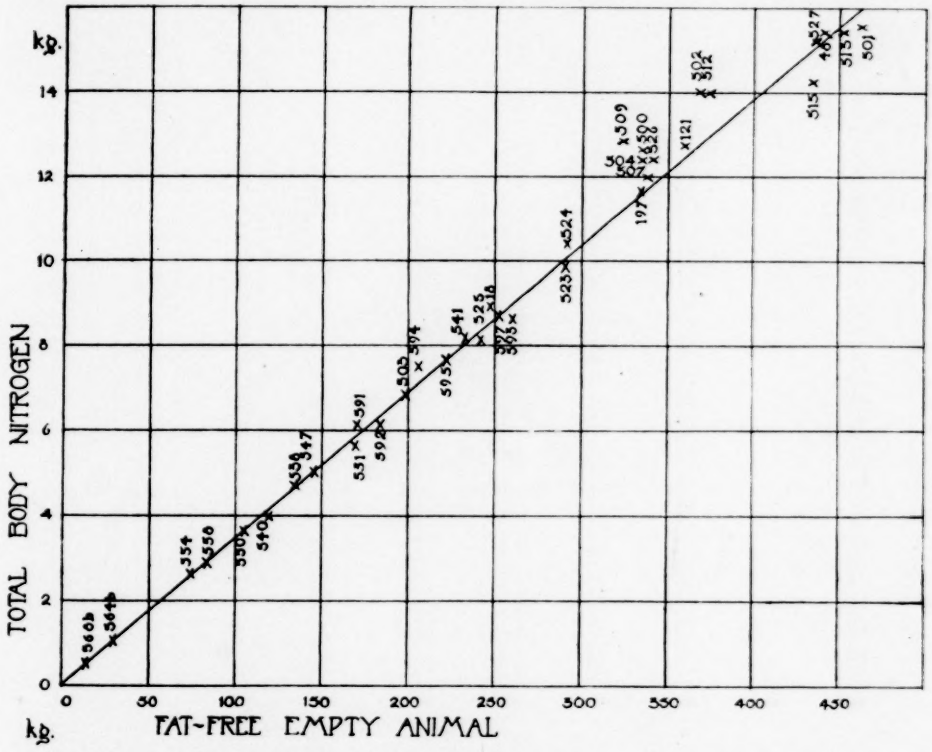

FIG. 2. Relation between total body nitrogen and fat-free empty animal.

The other animals were assigned to the groups defined according to their relative condition. The calves at birth are distinguished by a letter *b* after the number. It is the Group I animals 2 years old and over that show the decreasing proportion of fat-free weight and increasing proportion of fat. It is thus made evident that, while the unit of mass may be fairly uniform for the thin and medium fleshed animals and may represent about the same amount of active machine, this unit of mass has a different composition as the animal gets fatter.

In order to show that the fat-free empty weight is a good measure of the active machine there has been plotted in Fig. 2 the relation between the total nitrogen by analysis and the fat-free empty weight. This relation is very constant, 1 kg. of body nitrogen representing 29 kg. of fat-free animal irrespective of age, condition, or weight. In Fig. 3 the same relation is shown between the total nitrogen excluding that in the blood and the fat-free empty weight. 1 kg. of nitrogen exclusive of that in the blood

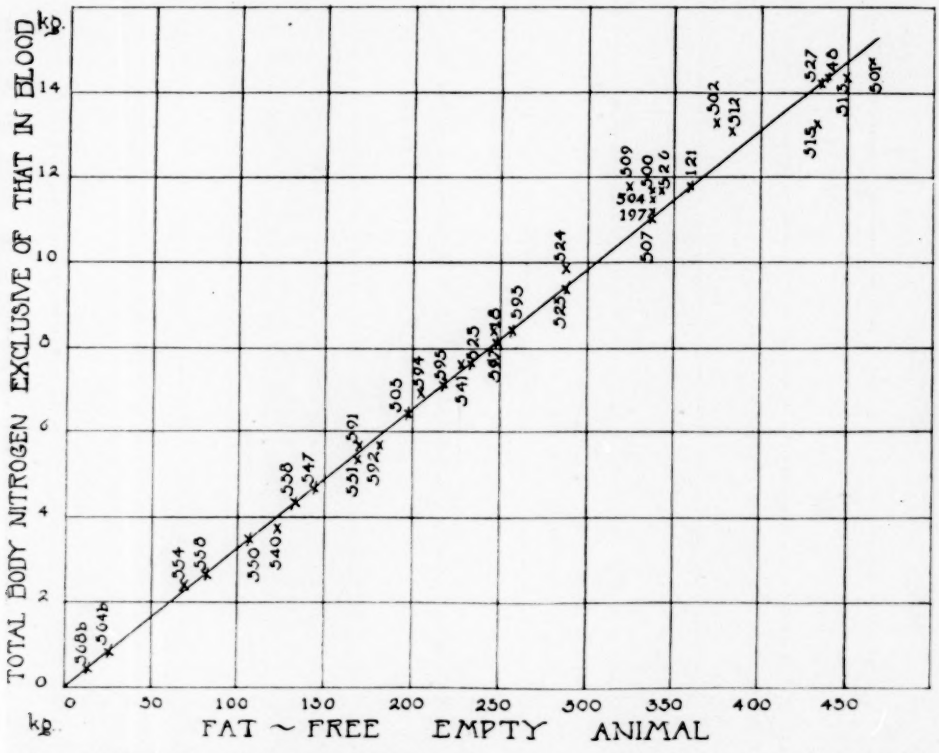

FIG. 3. Relation between total body nitrogen exclusive of that in the blood and fat-free empty weight.

represents 30.5 kg. of fat-free animal. The relation between the nitrogen excluding that in the blood, or hide, or bones, or any combination of the three, and the fat-free empty weight could be shown here. The relation is just as constant as those shown, one unit of nitrogen representing a larger unit of fat-free weight as more nitrogen is excluded from the total. The reason for excluding any nitrogen would be on account of its being inactive in the body metabolism. Since we have no standards of activity for the different divisions of the nitrogenous tissue the author has

considered the total nitrogen to be a good measure of the total active protoplasmic tissue.

Relation of Surface Area to Body Weight.

A better conception of the basal needs of animals for food can be obtained from a comparison of the relative surface areas of the animals. Since Rubner[2] and Richet[3] presented evidence to show that the heat production of living animals was propor-

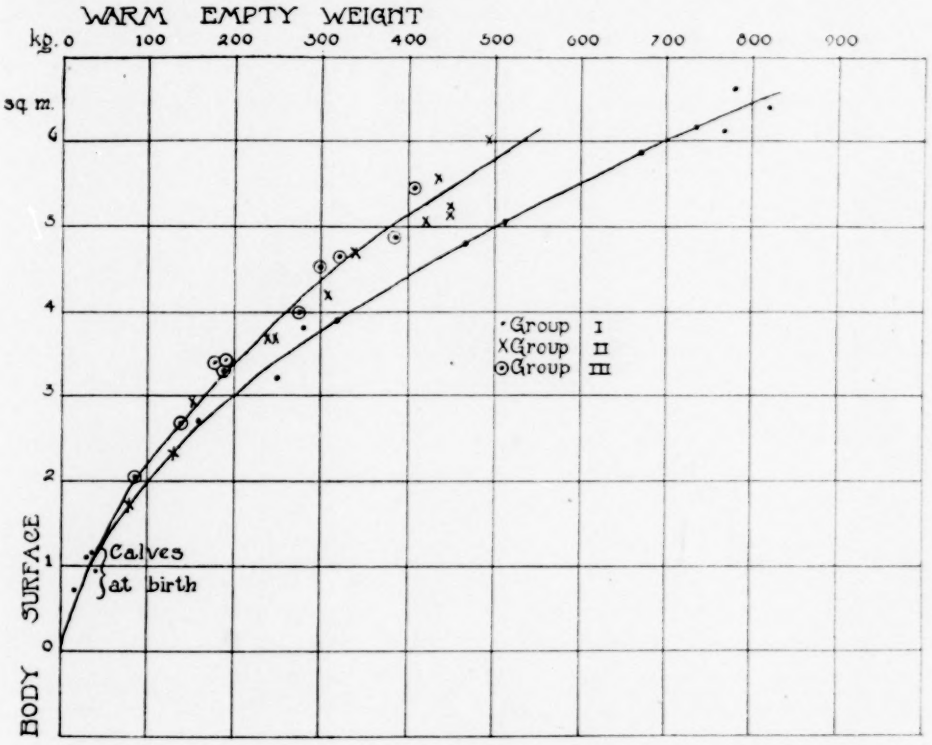

FIG. 4. Relation between body surface and warm empty weight.

tional to the body surface, this has been a much used unit of reference. The relation between body weight and surface area is derived from the mathematical law that the surfaces of like geometrical figures are proportional to the two-thirds power of the volumes. The specific gravity of like animals being about constant, it develops that $S = KW^{\frac{2}{3}}$, where S is the surface area

[2] Rubner, M., *Z. Biol.*, 1883, xix, 545.
[3] Richet, C., *Arch. de physiol.*, 1885, xvii, pt. ii, 284.

and W the body weight. The value of K has been worked out for a number of animals from a few more or less limited observations. The author[1] has compared actually observed values of S and W in 35 steers, and has found that K varies from 7.32 to 10.48 according to the fatness of the animal. For Group III animals it averaged 9.92, and for Group II animals 9.37. In Group I animals two classes were found; the young animals showed an average value for K of about 8.57, while the older animals showed an average value of 7.65. The relations found

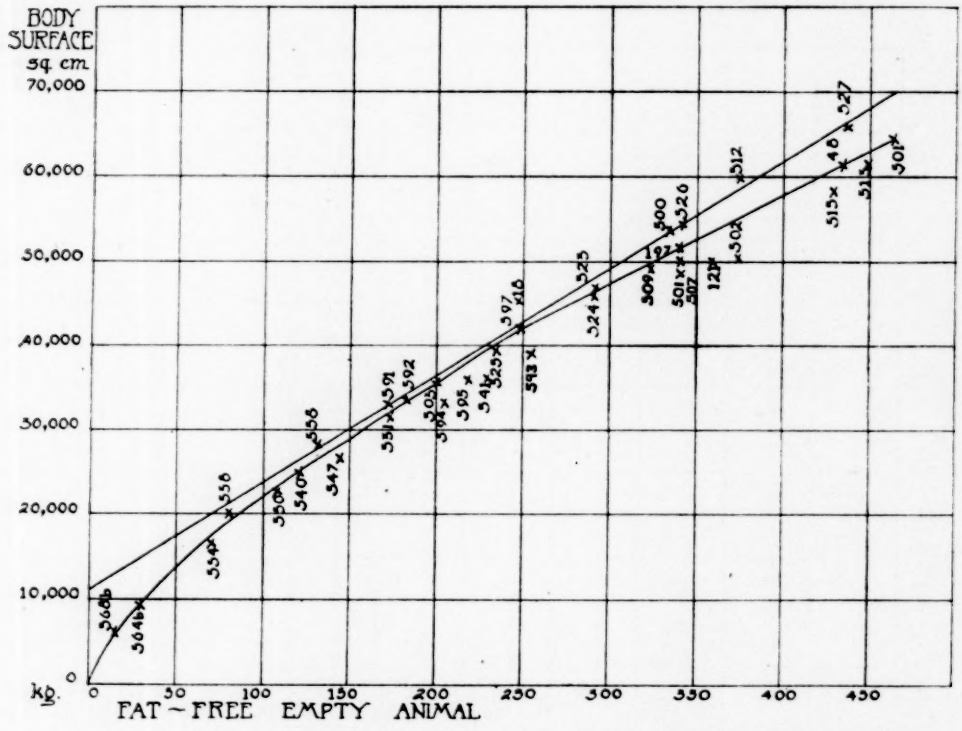

FIG. 5. Relation between body surface and fat-free empty weight.

are shown in Fig. 4, where one curve represents the relation for the animals in thin or fair condition, and a second curve the relation for the fat animals. The empty weight is used in place of the live weight in order to eliminate the influence of the amount of food and food residues upon the weight. The four calves at birth were considered to be representative of all groups at birth. The curves have the general appearance of power function curves.

The relation between surface area and fat-free weight is shown in Fig. 5. The straight line is drawn to show that the function

is by no means a straight line function, as it is in the case of nitrogen and fat-free weight. There are not shown here, however, two different curves, the effect of the fat animals being simply to cause a wider dispersion of values toward the right hand end of the curve.

In order to determine what relation exists between surface area and body weight recourse can be had to logarithmic cross section paper. Fig. 6 shows the values found for surface area and empty weight of 37 cattle. If we disregard the values for the Group I cattle, it is seen that the points arrange themselves well along a

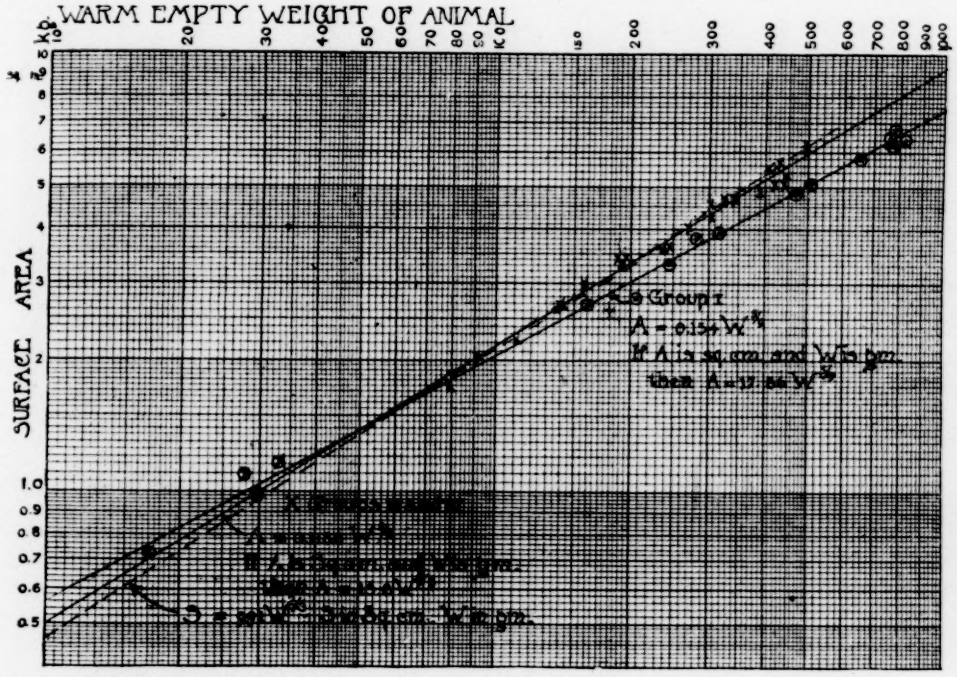

FIG. 6. Relation between surface area and warm empty weight plotted on logarithmic cross section paper.

definite straight line. Such straight lines on logarithmic paper are an indication of a power function of the general formula $Y = KX^n$. The inclination or tangent gives the value of n. If the line is followed to the point where it crosses the ordinate arising from the abscissa $X = 1$, K can be read off directly, or it can be calculated at the point where $X = 10$ or $X = 100$. The formula for the curve was thus found to be $Y = KX^{\frac{2}{3}}$. If A is the surface area in square meters and W the empty weight in kg., $A = 0.1186\ W^{\frac{2}{3}}$. The formula used at present is $S = KW.^{\frac{2}{3}}$ The

CORRECTION.

In Vol. XXIV, No. 3, March, 1916, on pages 305, 306, and 313, for $A = 0.134\ W^{\frac{2}{3}}$ read $A = 0.158\ W^{\frac{2}{3}}$.

exponent $\frac{2}{3}$ is $\frac{16}{24}$, and the exponent $\frac{5}{8}$ is $\frac{15}{24}$. This is a difference of only $4\frac{1}{6}$ per cent. However, the line $S = KW^{\frac{3}{4}}$ does not fit the facts shown. The dotted line, which was drawn at the inclination of $\frac{2}{3}$ through the greater part of the points, lies well below all the calves at birth and above most of the old animals. It does not divide the points as uniformly as does the line $A = KW^{\frac{5}{8}}$.

For the fat cattle (Group I) the difference between the observed formula and the one in present use is greater yet, for Fig. 6 shows the formula $A = 0.134\ W^{\frac{5}{8}}$. This shows a smaller surface area

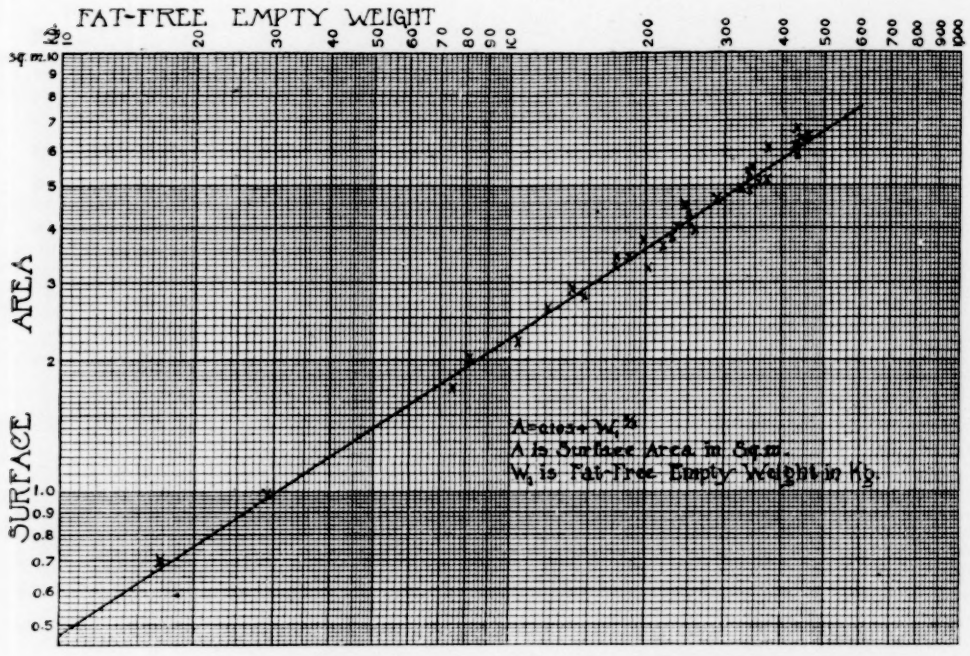

FIG. 7. Relation between surface area and fat-free empty weight plotted on logarithmic cross section paper.

relative to the weight for very fat cattle and is precisely the situation expected. Fatter animals have a rounder conformity and thus a smaller surface area for a given volume. The formula $S = KW^{\frac{2}{3}}$, then, does not fit the facts for very fat animals, and a large error will be introduced by using it for beef cattle.

Fig. 7 shows the data for surface area and fat-free empty weight treated in a similar manner. At a given weight the Group I animals gravitate toward the bottom of the values shown, while the Group II and Group III animals are toward the top.

But since the differences are slight and some errors are to be expected (see below), all the values are treated together. The points are distributed with considerable uniformity along a line at an inclination of $\frac{2}{3}$. The formula then in the same units is $A = 0.1034\ W_1^{\frac{2}{3}}$, where W_1 is the fat-free empty weight. On the basis of the fat-free animal the relation $S = KW^{\frac{2}{3}}$ does hold, and for beef animals containing a negligible amount of fat the usual formula can consequently be used without appreciable error.

Methods.

A critical discussion of the methods of obtaining the data plotted in Fig. 6 may be of service in showing the reliability of the conclusions. The cattle were weighed immediately before killing. They were then stunned, hoisted by the hind feet, and thoroughly bled into tared containers. The bleeding was assisted by pumping the fore legs. Blood dripping from the carcass later was caught and weighed. The cattle were then skinned. After disembowelling, the stomachs, intestines, and bladder were weighed full and then emptied, cleaned, and weighed again. The contents thus found for the stomachs, intestines, and bladder were subtracted from the live weight to get the warm empty weight. Errors of any moment could hardly creep in here. The entire hide, or sometimes the half hide from one-half of the carcass, which had been split by an expert, was traced on a large sheet of paper made by pasting heavy glazed wrapping paper together. The hide could be made to lie very flat and, with a little care, an accurate tracing could be made. Later these tracings were measured. The earlier measurements were made by drawing rectangles and triangles on the hide map. Later maps were cut into rectangles and triangles and fitted into a large rectangle the area of which could be easily determined. The latest measurements were made directly on large sheets of cross section paper upon which the hide outline had been traced. Of the errors due to the different methods used little can be proved except in the case of the last. Here two different cross section papers were used which caused the author to note discrepancies in the variation of K calculated from $S = KW^{\frac{2}{3}}$. These variations were expected to follow certain rules. The writer then checked up the

cross section paper and found that the second lot used was correct, but that the first lot gave an area too low by at least 5 per cent. The writer can thus predicate errors of like or even greater size due to some of the earlier methods used. Unfortunately these errors cannot be measured, as the meter sticks used were not kept or standardized. The writer feels that errors of as much as 5 per cent, or even 10 per cent, might have crept into the determinations of surface area. These could easily account for nearly all the points lying off the curves shown in Figs. 4, 5, 6, and 7.

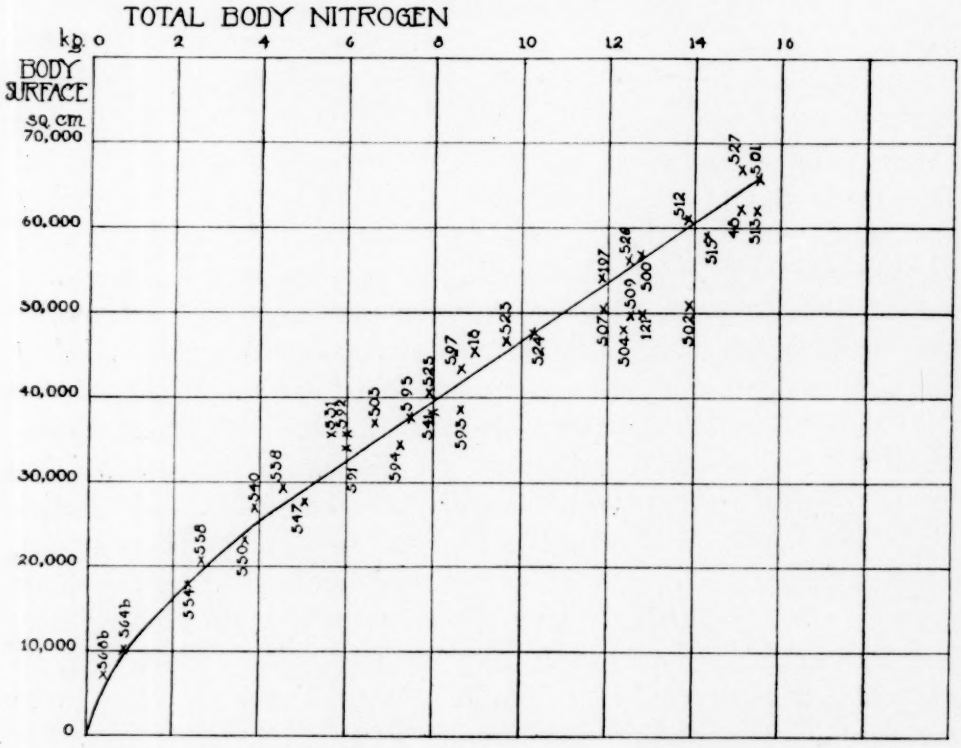

FIG. 8. Relation between body surface and total body nitrogen.

The Relation of Surface Area to Body Nitrogen.

It has just been shown that there is a relation between the fat-free empty weight and surface area of cattle, and it was shown earlier that the fat-free animal had a constant percentage of nitrogen. It should, therefore, be expected that a relation exists between the surface area and total nitrogen in the body. The values found are plotted in Fig. 8. The points are arranged uniformly along the curve shown. Fig. 9 shows the same relation

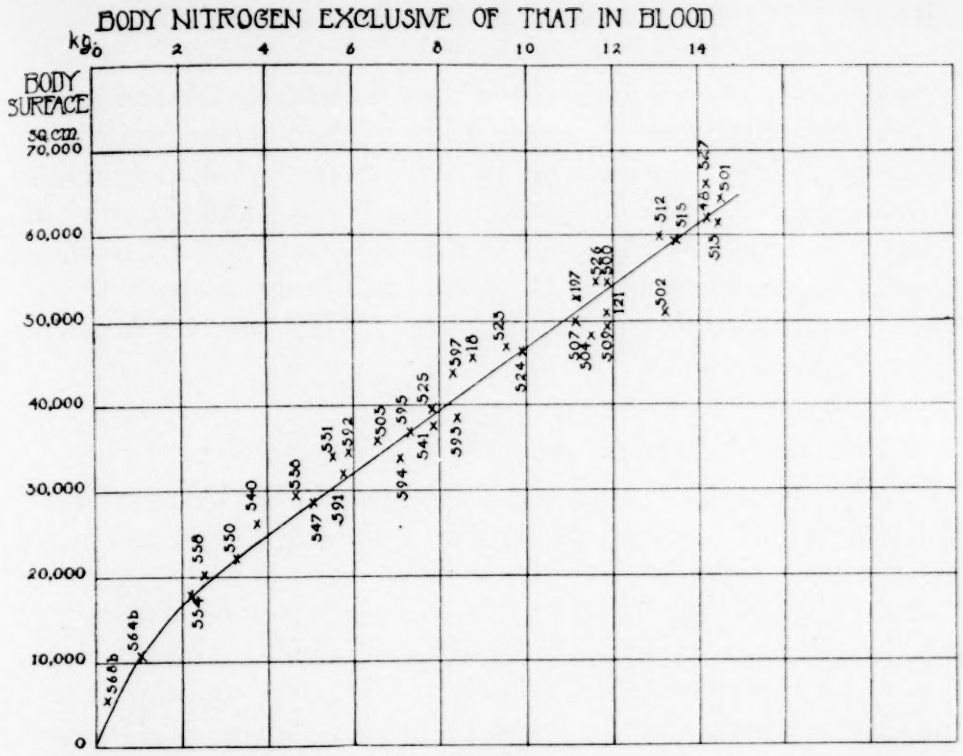

FIG. 9. Relation between body surface and total body nitrogen exclusive of that in the blood.

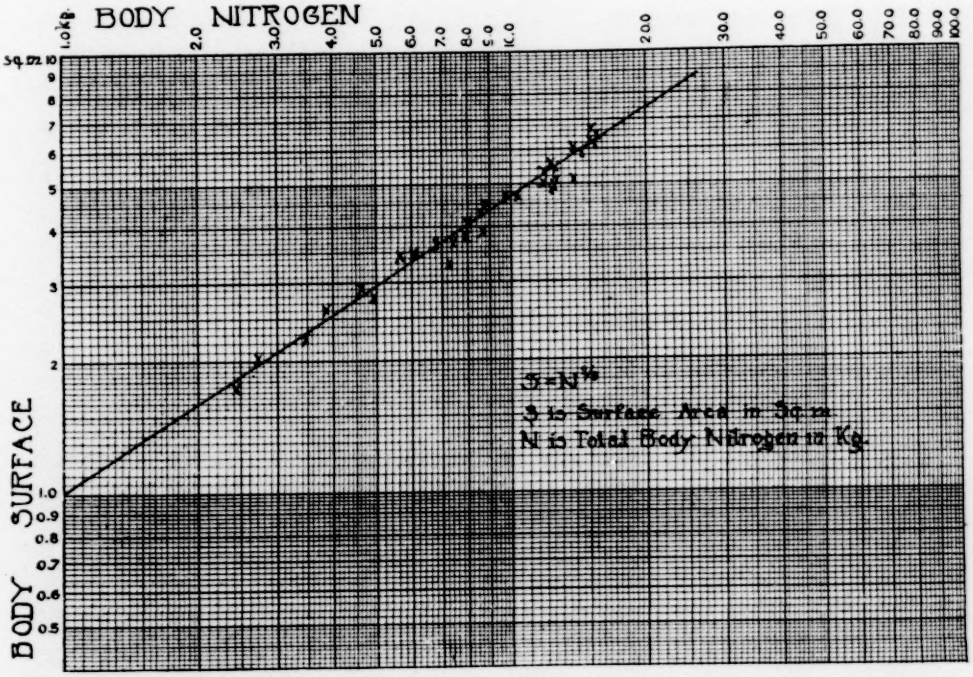

FIG. 10. Relation between body surface and total body nitrogen plotted on logarithmic cross section paper.

between body surface and body nitrogen exclusive of that in the blood. Practically no change is made in the relative position of the points. The values of Fig. 8 are shown plotted on logarithmic paper in Fig. 10. The inclination of the line is $\frac{2}{3}$ and the value of K is 1. So the formula found is $S = N^{\frac{2}{3}}$ where S is the surface area in square meters and N is the total body nitrogen in kg. This is a confirmation of the curve $A = KW_1^{\frac{2}{3}}$, as the relation of N to W_1 is constant.

The Relation of Blood to Body Weight.

The tissues and organs are dependent upon the blood for food. A larger animal has more blood than a smaller animal and has a greater need for food. In order to see if a unit of body requires a definite blood supply for its uses, a study will now be made of the relations of blood supply to body weight. Fig. 11 shows the relation of blood weight to empty weight. The three groups of animals are distinguished by different marks. There is a marked tendency for the points to arrange themselves along a line but with rather large variations from an average. Four of the oldest and fattest Group I animals line up along the dotted line far to the right of the average curve. This shows that for the very fat animals the blood supply does not keep pace with the body weight. This is to be expected since there is a large proportion of fat in these animals and the blood supply for the fatty tissue is small. Fig. 12 shows the relation of blood supply to fat-free empty weight. The relation is more nearly constant, 1 kg. of blood supplying 17.6 kg. of fat-free animal. However, the very fat animals lie above the curve along the dotted line, showing a greater proportion of blood to fat-free weight.

The Relation of Blood to Body Nitrogen.

Fig. 13 shows the relation of blood supply to body nitrogen. The three groups are distinguished by different marks. Most of the values are arranged along a straight line showing that 1 kg. of body nitrogen excluding that in the blood needs 1.6 kg. of blood to feed it. The very fat Group I animals again show the presence of relatively more blood for each unit of body nitrogen (protoplasmic tissue) to be supplied. Fat living seems to result

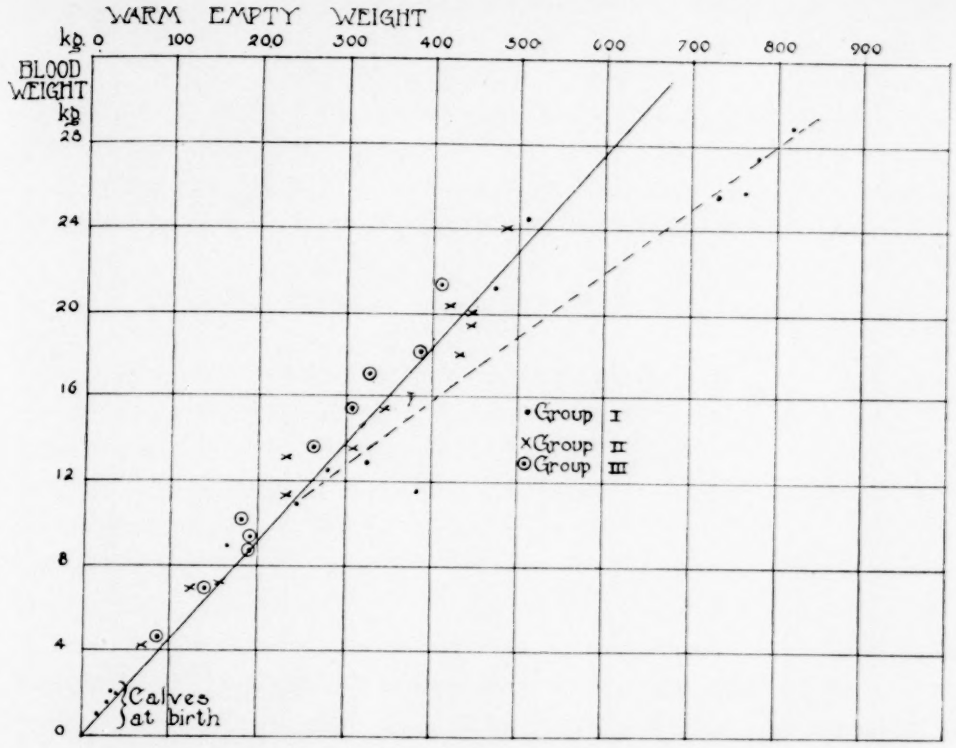

FIG. 11. **Relation** between blood weight and warm empty weight.

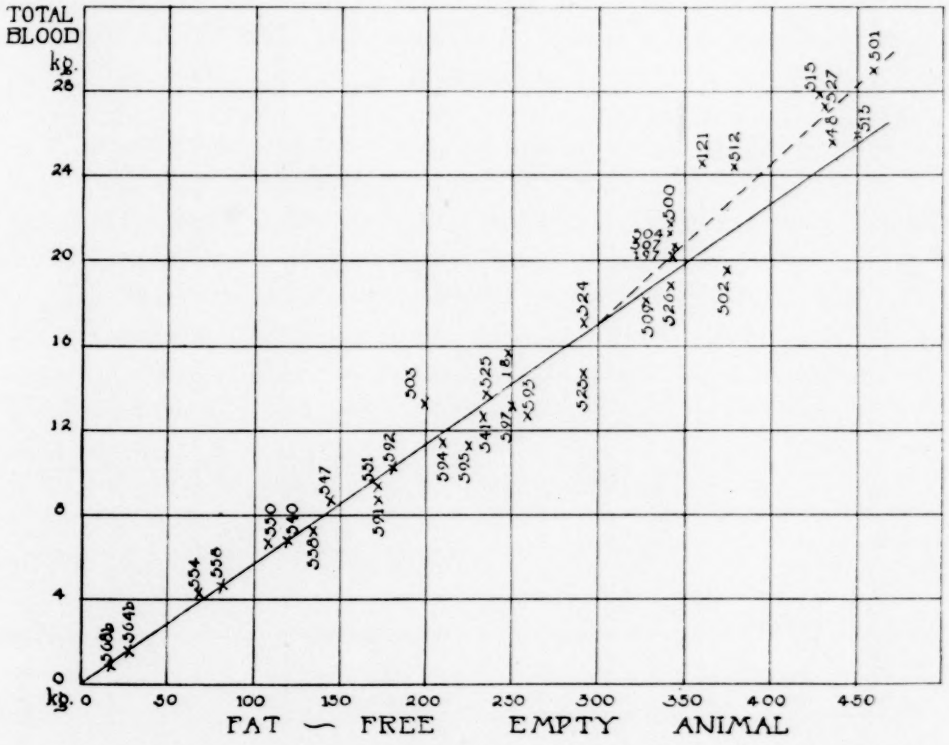

FIG. 12. Relation between blood weight and fat-free empty weight.

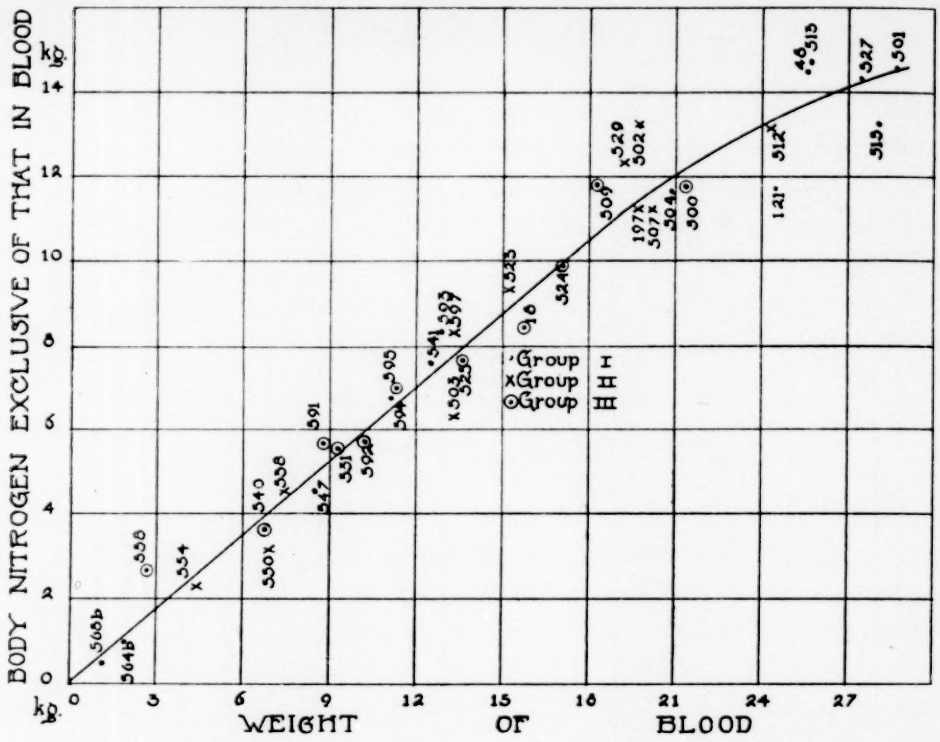

FIG. 13. Relation between total body nitrogen exclusive of that in the blood and blood weight.

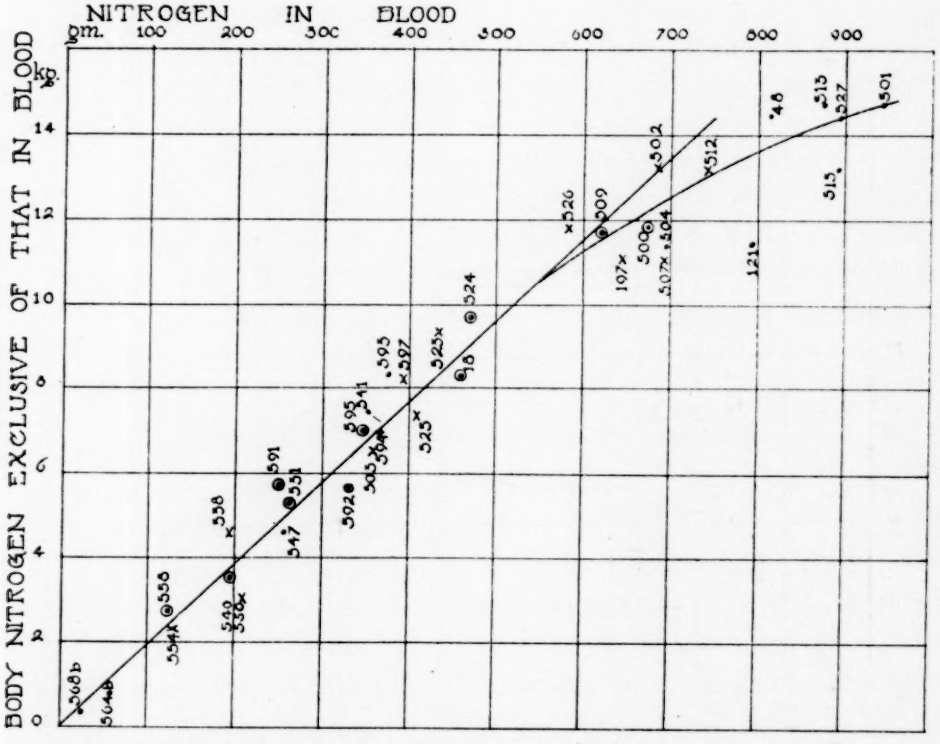

FIG. 14. Relation between total body nitrogen exclusive of that in the blood and nitrogen in the blood.

in more blood. The same relation is shown when the nitrogen in the blood is plotted against the body nitrogen (Fig. 14). All the Group I animals 2 years old and over show a greater proportion of total nitrogen in the blood to body nitrogen (exclusive of that in the blood) than do the other animals.

The Relation of Blood to Body Surface.

It has been shown that the body surface is a power function of the body weight and of the fat-free body weight, and that the relation of blood to fat-free body weight is simple and fairly constant. There should then be a relation between body surface and total blood. Fig. 15 shows the values plotted on ordinary cross section paper where a curve similar to the other power functions is shown. Fig. 16 shows the values plotted on logarithmic cross section paper. Here we find a straight line with the inclination of $\frac{5}{8}$. When S is the surface area expressed in square meters and B is the blood weight in kg., the formula is $S = 0.8\ B^{\frac{5}{8}}$. The dotted lines were drawn to show a band which would include practically all the points and thus aid in fixing the general inclination.

SUMMARY.

A simple relation has been shown to exist between the total body nitrogen and the fat-free empty weight of beef cattle. A simple relation exists also between blood weight and fat-free empty weight or between blood weight and total body nitrogen.

The surface area of beef cattle is a power function of the warm empty weight, the formula for fat animals being $A = 0.134\ W^{\frac{6}{9}}$ and for other animals $A = 0.1186\ W^{\frac{5}{9}}$. The surface area is also a power function of the fat-free empty weight. The formula for all animals is $A = 0.1034\ W_1^{\frac{3}{3}}$.

The surface area is a power function of the total body nitrogen, the formula being $S = N^{\frac{3}{3}}$.

The body surface and total blood are related in a similar manner, the formula being $S = 0.8\ B^{\frac{8}{8}}$.

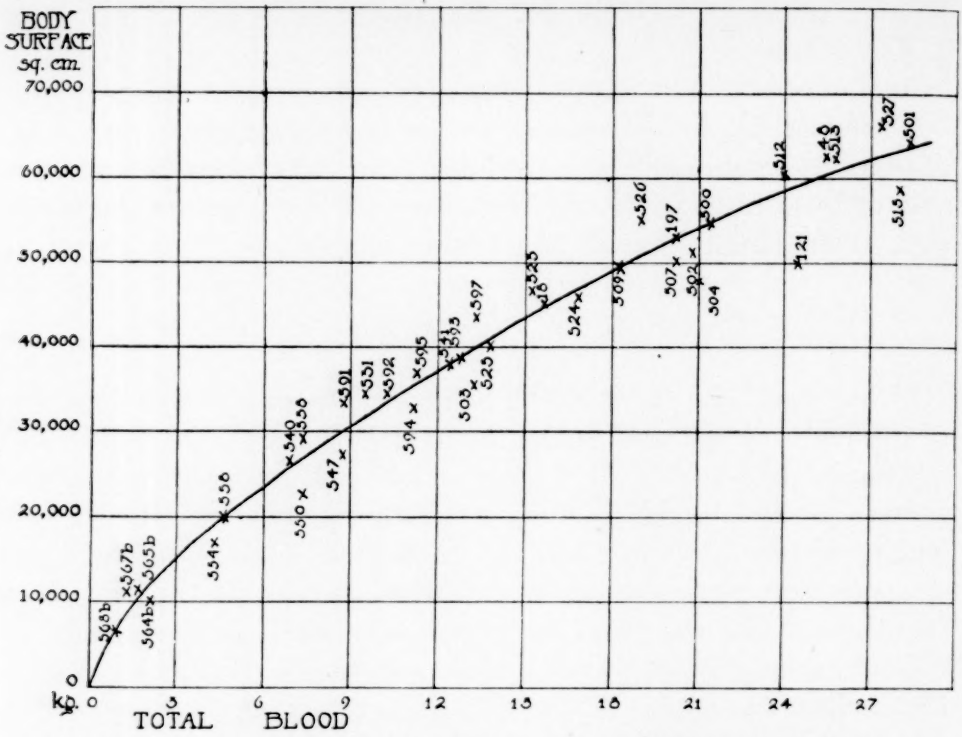

FIG. 15. Relation between body surface and blood weight.

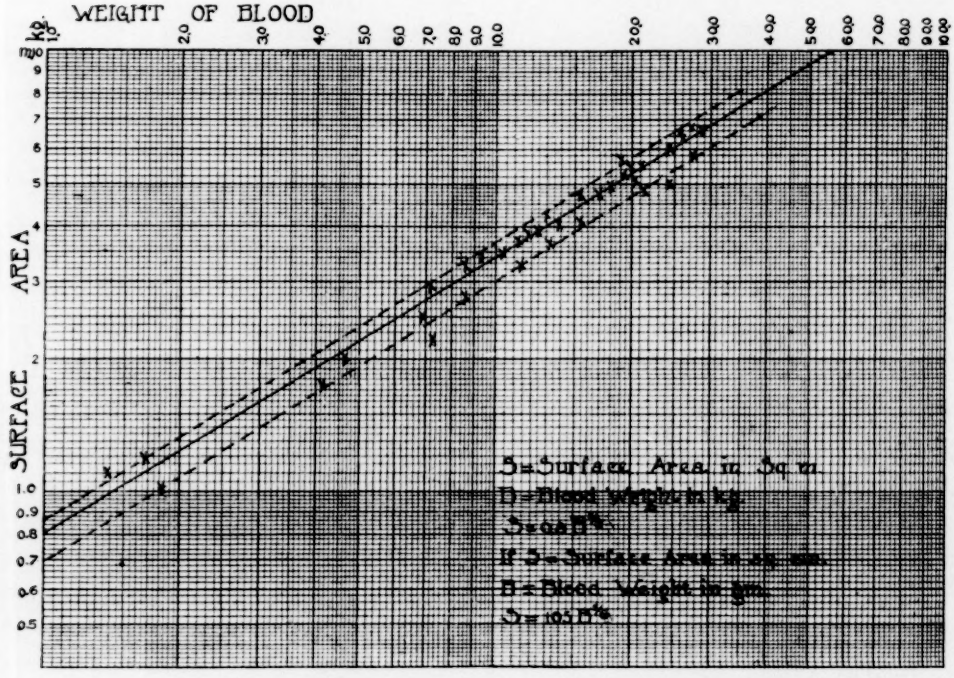

FIG. 16. Relation between body surface and blood weight plotted on logarithmic cross section paper.

CONCLUSIONS.

Since these relations have been shown to be fairly constant for animals under different conditions, it would seem to make little or no difference what unit of reference is used as a measure of basal metabolism as long as the unit used has been actually measured. It would seem also that for beef cattle the desired unit of reference could be calculated with considerable accuracy if the fatness of the animal and its condition with regard to fill can be determined. In many cases, however, the amount of fill in the beef animal can be disregarded. The author[1] has shown average variations in fill of 2 to 4 per cent of the live weight for mature animals of the three different groups above defined.

II.

In order to throw further light upon the question as to what unit of reference can best be used for basal metabolism and to test the validity of the conclusions given above, the maintenance data collected from a study of some of the animals used above can be discussed.

In the Research Bulletin referred to above[1] the data are given in some detail for 19 different animals for a total of 27 maintenance trials. The maintenance periods of some of the animals resulted as a consequence of treatment given for a purpose other than the study of maintenance. For other animals the units of reference had to be calculated and also the consumption of digestible nutrients from data obtained upon other animals. In order to exclude variations due alone to these factors some of the animals will not be included in this study. Most of the animals were upon maintenance at a body weight or body condition different from that found at the time of slaughter. Table I shows what factors were used in calculating the various weights and measures used. For example, Steer 507 was slaughtered at the end of the maintenance trial and the units of reference for the maintenance data were actually measured values at the maintenance weight. For Steer 48 the digestion factors used were obtained from the animal itself while on maintenance. The other units were calculated from the percentages found for Steer 18 since these animals were very much alike. The surface area

TABLE I.

Animal No.	Digestion factors.	Empty weight.	Weight of nitrogen.	Weight of blood.	Surface area.	Weight at which data were taken.
500	Group III.	Own percentage.	Group III average.	Own percentage.	Own K.	62 kg. heavier.
500	" III.	" "	" III.	" "	" "	33 " "
502	Own.	" "	Own percentage.	" "	" "	50 " "
507	"	Actual.	Actual.	Actual.	Actual.	Same weight.
509	"	Own percentage.	Own percentage.	Own percentage.	Own K.	50 kg. heavier.
512	Group II.	" "	" "	" "	" "	65 " "
512	" II.	Actual.	Actual.	Actual.	Actual.	Same weight.
524	" III.	Own percentage.	Own percentage.	Own percentage.	Own K.	30 kg. heavier.
526	" II.	" "	" "	" "	" "	87 " "
18	Own.	Actual.	Actual.	Actual.	Actual.	Same weight.
48	"	Percentage of No. 18.	Percentage of No. 18.	Percentage of No. 18.	Curve.	30 kg. heavier.
121	No. 18.	" " " 18.	" " " 18.	" " " 18.	"	Weight same as No. 18
164	Own.	Own percentage.	No. 121.	Own percentage.	"	Double maintenance weight.
197	"	Actual.	Actual.	Actual.	Actual.	Same weight.
588	"	"	Group I, 2 yr.	"	Curve.	" "
589	588 and 197	Own percentage.	" I, 2 "	Own percentage.	"	134 kg. heavier.
595	Own.	Actual.	Actual.	Actual.	Actual.	Same weight.
597	No. 595.	"	"	"	"	" "

was read off from Fig. 6, using the average curve for the Group II and Group III cattle.

Fig. 17 shows the total daily energy consumption for the different animals for body weight varying from 240 kg. to about 500 kg. It is seen that the energy consumption increases with the body weight, but not proportionally, and there are large variations. The points enclosed within circles indicate the animals for which the digestion factors were assumed from data on other animals.

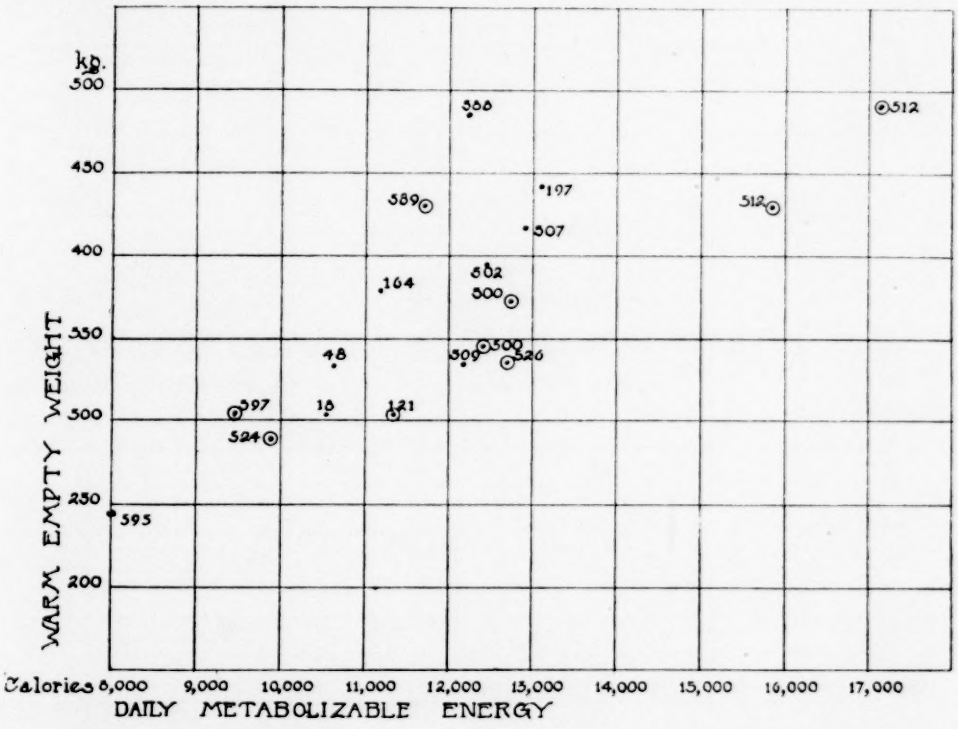

FIG. 17. Comparison of warm empty weight and total daily energy consumption for 16 beef cattle.

In Fig. 18 there is shown the heat consumption per kg. of body weight plotted against the body weight. The variations are great, a 500 kg. steer consuming from 25 to 35 calories per kg. There is a marked tendency for the consumption of energy per kg. to decrease with increasing weight. The variations from the average are 23.5 per cent.

In Fig. 19 are shown the values for heat consumption per square meter of body surface plotted against body weight. A tendency is shown for the heat consumption per unit of surface area to in-

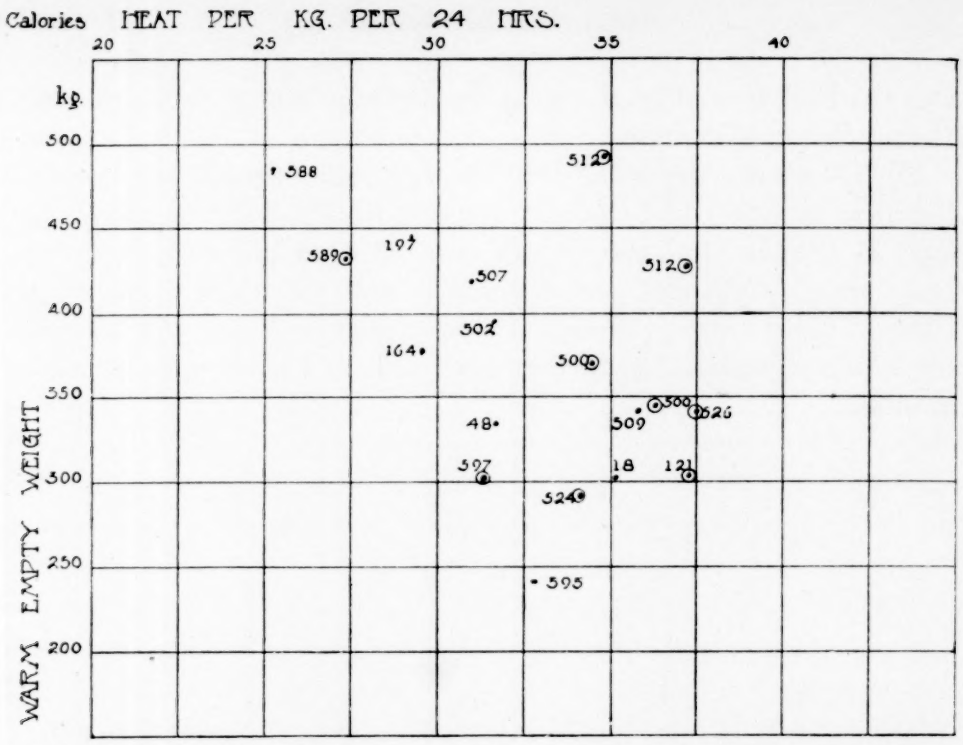

FIG. 18. Comparison of warm empty weight and daily heat consumption per kg. of body weight.

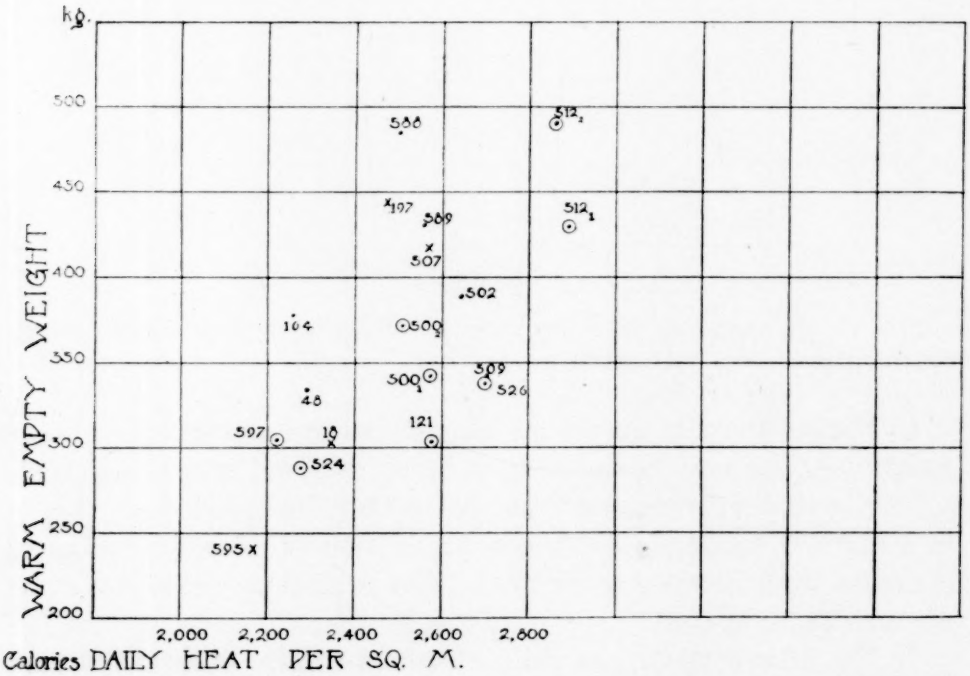

FIG. 19. Comparison of warm empty weight and daily heat consumption per square meter of body surface.

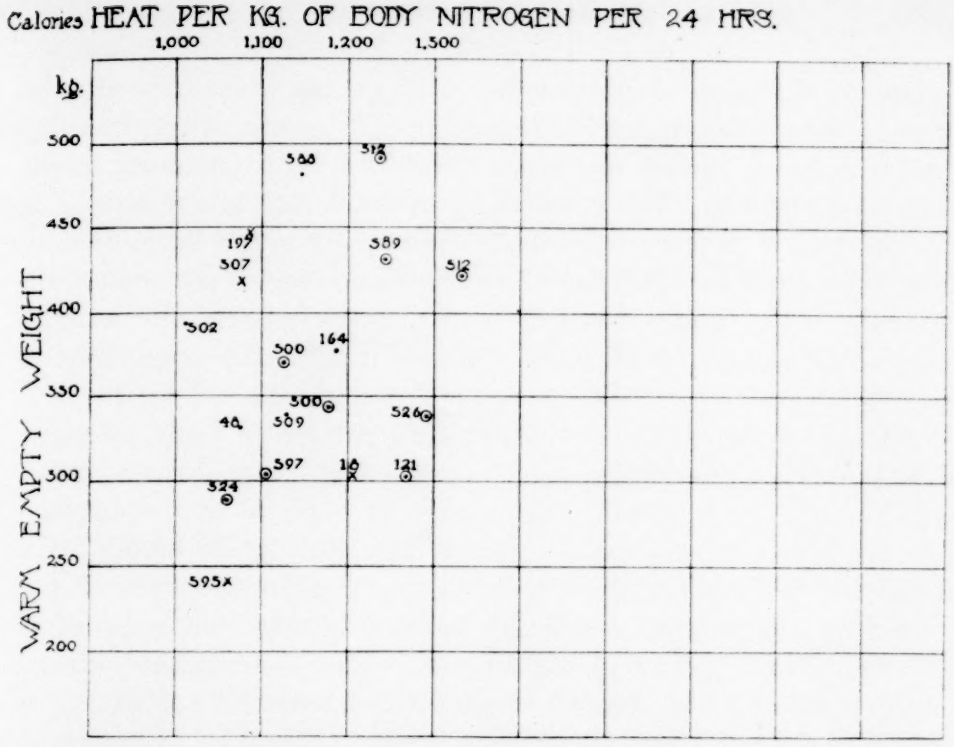

FIG. 20. Comparison of warm empty weight and daily heat consumption per kg. of body nitrogen.

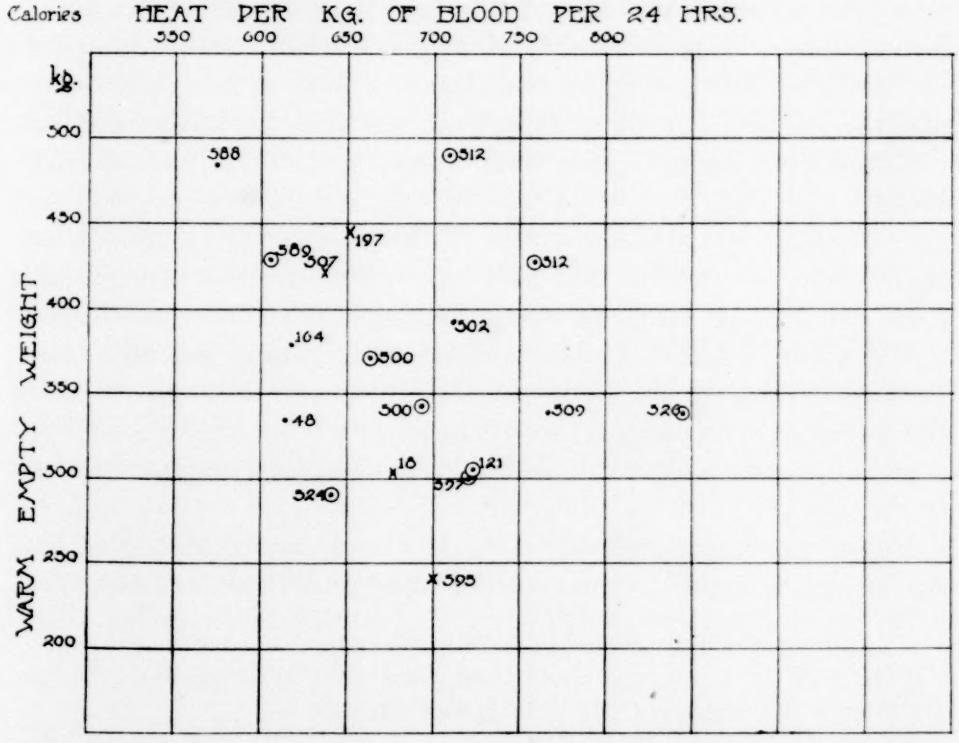

FIG. 21. Comparison of warm empty weight and daily heat consumption per kg. of blood.

crease as the body weight increases. This has been shown[1] to be due to the greater weight sustained by the animal while standing and walking. The energy required for this is proportional to the weight sustained. The greatest variation from the average value in Fig. 19 is 15.6 per cent with one animal strikingly high. Omitting the values for this steer the variations are 11.3 per cent of the average. The points marked with a cross indicate the animals for which all the data were obtained under the conditions of maintenance.

Fig. 20 shows the heat consumption per kg. of body nitrogen. The points are distributed more nearly along a vertical line or vertical band. The extreme variation is 14 per cent of the average.

Fig. 21 shows the heat consumption per kg. of blood. The tendency is for a slight decrease in this consumption per unit of blood as the animal gets heavier. An extreme variation of 15 per cent from the average is shown. The steer at the extreme right seemed to be abnormal with regard to the percentage of blood in the body, and is therefore omitted.

Several factors have been shown to enter into the consideration of the cause of the variations shown above. These were discussed at length in the Bulletin already referred to. Since it can be assumed that they affected similarly the heat consumption per the different units of reference, the validity of the above comparisons should not be questioned. On this basis the smallest variations are shown in the heat consumption per unit of body surface and the greatest variations in the heat consumption per unit of body weight. Per unit of body nitrogen (protoplasmic tissue) and per unit of blood the variations are not much larger than per unit of body surface. If there is a choice of units it would seem to fall upon the surface area. Since this can easily be calculated from the body weight by the formulas proposed by the writer of this paper, it would seem that this unit fits well the needs of the investigator in animal nutrition, especially with beef cattle.

These conclusions are opposed to the views of Benedict[4] but are in agreement with those of Du Bois[5] and Graham Lusk.[6]

[4] Benedict, F. G., *J. Biol. Chem.*, 1915, xx, 279.

[5] Du Bois, E. F., *J. Am. Med. Assn.*, 1914, lxiii, 827. Du Bois, D., and Du Bois, E. F., *Arch. Int. Med.*, 1915, xv, 868.

[6] Lusk, G., *Science*, 1915, xlii, 816.

THE ELECTROLYTIC DETERMINATION OF IODINE PRESENT IN ORGANIC MATTER.

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In a previous paper[1] a method for the determination of the iodine present in tissues or other organic matter was described in detail. This method consists essentially of a fusion, reduction of any iodate formed, careful extraction of the iodides, precipitation with palladium chloride, and the estimation of the palladous iodide formed by colorimetric comparison with a standard solution of that substance.

This method has as a matter of practice been entirely satisfactory. It is far more sensitive than any method previously used and it is free from the objectionable features of several of the earlier procedures. As our immediate interest in the matter rested on the determination of iodine in tuberculous tissues,[2] obviously very complex, and in amounts frequently so small as to be barely perceptible by even this most sensitive method, it has seemed desirable to check the procedure in every possible way. I have therefore developed an electrolytic method of analysis to cover the same ground.

As the method already published involved the colorimetric determination of the palladous iodide formed in the reactions, so this further development has resolved itself into the problem of estimating the palladous iodide electrolytically. The amount of palladium and the amount of iodine are both determined, checking each other by calculation. This method is far less sensitive than the colorimetric determination. It can be used advantageously as a direct method of analysis if the amount of iodine

[1] Krauss, R. B., *J. Biol. Chem.*, 1915, xxii, 151.

[2] Lewis, P. A., and Krauss, R. B., *J. Biol. Chem.*, 1915, xxii, 159.

involved is relatively large, ± 0.0005 gm. After a series of determinations by the colorimetric method each involving amounts of iodine much smaller than this, it is advantageous to collect all the material and repeat the analysis electrolytically, thus making certain that there has been no gross error.

The following observations by previous workers bear on the problems involved in the electrolytic method.

Whitfield[3] estimated iodine electrolytically proceeding from the silver salt, weighing the metal after electrolysis. Vortmann[4] has developed an electrolytic scheme for the estimation of pure potassium iodide. Specketer[5] estimates iodine by electrolytic methods, using graded potentials.

None of these methods are suitable for routine determinations. The quantities of chlorides and bromides present interfere with the first two; the low and absolutely constant voltage as well as the necessity for absolute exclusion of air and definite acid concentration makes the last unsuitable.

That palladium can be deposited from a solution of palladammonium chloride was found by Smith[6] and Keller.[7]

It is well known that palladous iodide affords a separation of iodides from chlorides and bromides, and that it is insoluble in water, alcohol, ether, and dilute hydrochloric acid.

Method.

The substance to be analyzed is fused and treated exactly as in the colorimetric method until the alcohol-acetone solution of iodide is obtained. This is evaporated to dryness, taken up in 100 cc. of water, and an excess of palladous chloride added. After the addition of one or two drops of dilute hydrochloric acid the whole is heated to boiling in a beaker.

The black palladous iodide is filtered off, using a vacuum filtering dome, an ordinary funnel, and hardened filter paper.

It is not necessary to remove all the iodide from the beaker, but after washing the beaker and the filter with water, the beaker is used as a receiver and the iodide on the filter dissolved with hot aqueous ammonia into the beaker.

[3] Whitfield, J. E., *Am. Chem. J.*, 1886, viii, 421.
[4] Vortmann, *Z. Electrochem.*, 1895–96. ii, 314.
[5] Specketer, H., *Z. Electrochem.*, 1897–98, iv, 539.
[6] Smith, E. F., *Am. Chem. J.*, 1891, xiii, 206; 1892, xiv, 435.
[7] Smith, E. F., and Keller, H. F., *Am. Chem. J.*, 1890, xii, 212.

All the palladous iodide remaining in the beaker is carefully dissolved. For small weights of iodide the solution is electrolyzed directly; for larger ones it is first made up to a definite volume in a flask and then an aliquot part electrolyzed.

With a weighed gauze platinum cathode, the solution is electrolyzed in a platinum dish at a dilution of 60 cc. and at 65°C. About 1 cc. of ammonia is added. The current may vary from 0.5 to 1 ampere, the voltage from 5 to 10 volts. Rotating the cathode at about 300 R. P. M., the palladium is readily deposited.

The current is interrupted, the cathode carefully washed with water over the dish, then after removal of the cathode from the spindle of the rotator, it is washed with water, alcohol, and ether. After warming in an electric oven and cooling in a desiccator, it is weighed. About 25 minutes are required for the complete deposition.

The solution is evaporated to half volume, with the addition of 1 cc. of 10 per cent sodium hydroxide and 1 gm. of sodium potassium tartrate, and is covered with a watch glass.

After cooling and diluting to 40 to 50 cc., the iodine is deposited on the silver electrode as anode, the current having been reversed. The pressure should be 2 volts, current 0.01 to 0.05 amperes, and the anode stationary. Time required, 30 to 40 minutes. Stirring at this point seems to favor the detachment of silver iodide. After washing as above, the electrode is dried at 300°C. in an electric oven. It is cooled and weighed.

All reagents must be carefully examined for chlorides, and the silver is best purified by electrolysis.

Preparation of Electrodes.

The silver plated anode is prepared as follows: A circular platinum gauze electrode is heavily plated from a silver ammonium oxalate solution, using 1 to 2 amperes and 5 to 10 volts and rotating at about 300 R. P. M. This electrode is washed with ammonia water, distilled water, and ignited. It is then boiled with hot water for half an hour, washed in water, absolute alcohol, absolute ether, dried at 300°C. in an electric oven, and weighed.

After each determination the silver iodide is removed by cyanide and the washing repeated. An electrode lasts from six to twelve determinations before replating is necessary.

The platinum cathode (circular, gauze) is freed from palladium by dipping into a mixture of saturated salt solution and chromic acid. This scarcely attacks the platinum.

The following tables show values obtained by this method.

In Table I are the figures obtained by electrolyzing a portion of a standard solution of palladammonium iodide prepared from a standard solution of potassium iodide.

Table II shows values obtained by use of the complete method on some typical organic iodine compounds.

Table III shows a check on a series of determinations by the colorimetric method of the iodine content of tuberculous and normal tissues.

TABLE I.

	Iodine.	Palladium.
	mg.	*mg.*
Present	2.50	1.054
Found	2.60	1.05
"	2.45	1.08
"	2.45	1.00

TABLE II.

Iodobenzoic acid 0.5 gm. taken. 51.21 per cent I. Dilution 500 cc.	Iodine.		Palladium.	
	Present.	Found.	Present.	Found.
cc.	*mg.*	*mg.*	*mg.*	*mg.*
1	0.512	0.50	0.216	0.20
5	2.56	2.50	1.08	1.10
10	5.12	5.08	2.16	2.11
Iodoacetanilide 0.5 gm. 48.65 per cent I. Dilution 500 cc.				
1	0.49	0.46	0.21	0.22
5	2.43	2.40	1.03	1.00
10	4.86	4.84	2.05	2.05
Diododiphenyl 0.5 gm. 62.56 per cent I. Dilution 500 cc.				
1	0.63	0.64	0.27	0.26
5	3.13	3.10	1.32	1.30
10	6.26	6.21	2.64	2.63

TABLE III.

Check on Twenty-two Colorimetric Determinations.

	Iodine.	Palladium.
	mg.	*mg.*
Colorimetric	7.016	Calculated..... 2.96
Electrolytic	6.90	Found..... 2.82

SUMMARY.

1. The electrolytic method may be used to estimate gravimetrically the iodine present in organic tissues or in organic iodides.

2. It provides an adequate check on a series of determinations by the palladous iodide colorimetric method.

3. In all cases the estimation of both cation and anion gives another check.

STUDIES ON THE THEORY OF DIABETES.

VI. THE BEHAVIOR OF *dl*-GLYCERIC ALDEHYDE IN THE NORMAL AND DIABETIC ORGANISM.

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Pure crystalline *dl*-glyceric aldehyde was administered by different routes to normal rabbits and guinea pigs and to completely phlorhizinized and glycogen-free dogs. The experiments continue an investigation reported 5 years ago,[1] and are probably the first in which the pure aldo triose has been studied in living animals. The results are discussed in their relation to the theory of metabolism and diabetes, which forms the theme of the present series of communications. Coincident with the work of establishing the behavior of *dl*-glyceric aldehyde in the diabetic organism, further observations were made upon the technique of deglycogenation by means of epinephrin, which was described in our last paper.[2] Epinephrin was given as before once every 3 hours subcutaneously, but the dosage was varied in different experiments. Further experimentation with different dosages in a series of experiments not here reported taken in conjunction with the present series lead to the conclusion that 0.04 mg. of epinephrin per kilo of body weight subcutaneously once every 3 hours nearly always suffices to eliminate all glycogen within the first 24 hours of the phlorhizin régime and exerts little if any harmful effect upon the animal. With smaller doses the time necessary for deglycogenation is unduly protracted, while larger ones—although capable of discharging the glycogen in 12 to 18 hours—are unnecessarily severe and frequently exert deleterious effects on the circulation and secretions.

[1] Woodyatt, R. T., *J. Am. Med. Assn.*, 1910, lv, 2109.

[2] Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1915, xxi, 1.

Further studies were also made of the character of the urinary retentions which so frequently develop in phlorhizinized dogs after the administration of certain classes of chemical substances, as dealt with in our paper on narcotic drugs.[2] The urinary suppressions which followed the administration of glyceric aldehyde were at first so marked that the urine gave no conclusive proof that the triose had been converted into glucose. But it was found that the suppressions could be minimized or annulled by an appropriate alkaline diuretic therapy based on the principle that the retentions were due to acid in the tissues, secondary to tissue asphyxia and provocative of processes akin to edema. It was then demonstrated that glyceric aldehyde may be converted almost quantitatively into glucose. The control of suppressions by the use of alkali and a diuretic salt solution supports the interpretations which were made in our last paper that the decreases in the glucose, nitrogen, and acetone body eliminations which were observed after the administration of ether, chloroform, and acetaldehyde were expression of retentions rather than primary metabolic changes. The effects of the alkaline diuretic treatment upon the suppressions which follow the administration of glyceric aldehyde suggest that other toxic retentions might be similarly managed and that by a suitable supplemental treatment of the animals the phlorhizin technique may still be used for testing the behavior of many substances which could not otherwise be given without so interfering with elimination as to make the results of urine analysis valueless. It is also considered that these results of therapy directed against toxic retentions in diabetic dogs have a certain clinical bearing.

HISTORICAL.

The chemical literature concerning glyceric aldehyde with particular reference to the technique of its preparation has been collected and enlarged in two articles by E. J. Witzemann[3] of this laboratory. The following points may be repeated, since they aid in the criticism of the biological experiments. Glyceric aldehyde was first isolated in pure form in 1898 by Wohl[4] by means of an elaborate synthetic method which, until 1914, remained the only means by which pure glyceric aldehyde could be

[3] Witzemann, E. J., *J. Am. Chem. Soc.*, 1914, xxxvi, 1908, 2223.

[4] Wohl, A., *Ber. chem. Ges.*, 1898, xxxi, 1796, 2394.

prepared. Before 1898 the actual properties of this triose were therefore not established, although as early as 1860 some of the more important had been prophesied correctly. In 1887 Grimaux, and Fischer and Tafel established the existence of trioses in syrups obtained by the oxidation of glycerol; and in 1897 Fenton and Jackson proved that much glyceric aldehyde was present in syrups left by the oxidation of glycerol with H_2O_2 in the presence of ferrous sulphate, but no crystalline glyceric aldehyde was ever separated from such syrups until this was accomplished by Witzemann in 1914.

With the growth of chemical interest in the trioses as hypothetical substances they began to be assigned rôles in schemes of animal sugar metabolism and particularly the aldo triose. But glyceric aldehyde has never yet been demonstrated in a normal body fluid or tissue, although this has been claimed erroneously on several occasions, and until 1904, no direct experiments had been made to establish its actual physiological properties. This was doubtless because the one known method of preparing it was and is time-consuming and difficult to a degree which makes its successful execution a chemical feat.

In 1904 Neuberg[5] stated that experiments had been made in conjunction with Blumenthal in which crude "glycerose," prepared by the oxidation of glycerol with H_2O_2 in the presence of a ferrous salt (Fenton and Jackson's method) had been administered to normal and diabetic men and animals. According to Neuberg, the glycerose was a "sweet colorless syrup" and "wholly non-toxic." When given to normal animals and men by different routes it was "completely oxidized even in very large doses." It was found to be a glycogen builder and the tolerance limit for it was "greater than for all artificial and most natural sugars including glucose." "Remarkable" was "the behavior of glycerose in the diabetic organism. . . ." "Here also it" was "completely burned without increasing the existent glycosuria in the least degree." As recently as 1913 Neuberg[6] has cited this report without reference to subsequent communications, from which it would appear that these statements have not been supplemented by a detailed description of experiments. They record, however, the first effort to test experimentally the effects of glyceric aldehyde in the body.

In 1910 one of us[1] published the results of experiments made with similar syrups. These syrups were faintly colored and sweet, with an aromatic flavor and reducing power equivalent to 50 to 60 per cent glucose solutions. Subsequent work has proved that they contain a large quantity of *dl*-glyceric aldehyde, which Witzemann succeeded in separating as the diethyl acetal and finally crystallizing, but since pure glyceric aldehyde is found to have the same reducing power as glucose, the total content of glyceric aldehyde in these syrups was never above 60 per cent. They were found not to be wholly non-toxic, as Neuberg and Blumenthal had held for theirs; and it has since been shown that crystalline *dl*-glyceric aldehyde is three

[5] Neuberg, C., *Arch. Anat. u. Physiol., Physiol. Abt.*, 1904, 571.

[6] Neuberg, *Oppenheimer's Handb. Biochem.*, 1913, 588.

times as toxic as glucose when given by mouth and six to eight times as toxic by parenteral routes. It was also noted that syrups of this kind when fed to cases of severe diabetes in dilute subdivided doses caused increases of glycosuria sometimes amounting roughly to upwards of 90 per cent of the total weight of the syrup given. In these cases there was no appreciable effect on the excretion of ammonia and acetone bodies (Table I). The essential features of this observation have since been confirmed with pure material. It was observed, however, that when the glycerose was given in relatively concentrated solutions and all at one time, there was little immediate increase of the glycosuria and in such cases there was a remarkable decrease in the output of ammonia and

TABLE I.

Abstract from the Protocol of a Case of Human Diabetes on a Constant Diet (January, 1909). On January 25, 26, and 27 the Patient Slowly Sipped Daily 40 Gm. of Glycerose Syrup in Dilute Solution Throughout the Waking Day, Thus Receiving 120 Gm. in 3 Days. The Periods Are of 24 Hours Each. The Unknown Glycogen Content Naturally Constituted a Source of Error.

Date.	Urine volume.	Glucose.	β-Hydroxybutyric acid.	Acetone.	Ammonia.	Nitrogen.	Remarks.
1909	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
Jan. 24	2,475	60.1	23.8	4.0	1.7	18	
" 25	**2,910**	**96.9**	**19.5**	**3.9**	**1.7**	**18**	3 glyceric aldehyde days during which together with the next 2 days the average glucose excretion was 32 gm. above the average for 5 days preceding, this increase corresponding roughly to 80 per cent of the glycerose given.
" 26	**2,700**	**110.5**	**18.0**	**3.9**	**1.6**	**18**	
" 27	**2,690**	**107.6**	**18.7**	**4.0**	**1.4**	**16**	
" 28	3,310	85.0	25.0	4.7	1.9	18	
" 29	3,340	80.0	26.0	4.7	2.0	18	
" 30	2,800	72.0	23.8	4.2	1.9	18	

acetone bodies. An example of such an effect is shown in Table II. At this time the idea was entertained that the more concentrated and acute dosages, producing higher concentrations of glyceric aldehyde in the tissues, favored direct burning and concomitant antiketogenesis, while the slow administrations, producing lower concentrations of glyceric aldehyde in the tissues, had permitted larger percentages to be transformed into glucose and so to escape oxidation. Such results appeared more in harmony with Neuberg's view. Later work would indicate, however, that the failures to obtain more marked increases of glycosuria after the heavy doses of glycerose were due at least in part to retentions induced by the toxic action of the glycerose on the kidneys and other tissues and that the

striking suppressions of the acetone body and nitrogen outputs were at least in part simultaneous expressions of the same process. These apparently favorable effects were accordingly questioned.[7]

In 1912 reports of experiments with glyceric aldehyde appeared from several sources. Parnas[8] perfused tortoise livers with solutions of *dl*-glyceric aldehyde syrups which he prepared in accordance with the method of Wohl. He found that if the perfusion fluid contained sufficient oxygen in addition to *dl*-glyceric aldehyde, glycogen was formed, but not otherwise. Smedley[9] added impure glyceric aldehyde solution to liver emulsions and noted that the triose disappeared without evidences of a new

TABLE II.

An Earlier Experiment on the Same Case. On January 19 the Patient Received by Mouth 75 Gm. of Glycerose Syrup in Lemonade, Which He Drank Slowly in the Course of an Hour.

Date.	Urine volume.	Glucose.	β-Hydroxybutyric acid.	Acetone.	Ammonia.	Nitrogen.	Remarks.
1909	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
Jan. 10–15	2,550	68	23	4.0	1.4	16	
" 16	2,170	65	19	3.8	1.3	17	
" 17	2,630	74	26	4.2	1.3	18	
" 18	2,650	72	22	3.9	1.6	19	
" 19	**2,500**	**87**	**5.3**	**1.6**	**0.8**	**17**	Glyceric aldehyde day with gross glucose increase of 17 gm. above the average of 8 preceding days. This corresponds to about 23 per cent of the weight of the syrup given.
" 20	2,360	54	24	3.5	1.1	17	
" 21	2,620	74	20	4.2	1.6	17	
" 22	2,850	74	25	4.6	1.8	17	
" 23	2,475	60	24	4.00	1.9	18	

[7] This same interpretation of a delayed urinary excretion of sugar and the acetone bodies as an improvement of the diabetic metabolism, still recurs in the literature with remarkable frequency. It has been made by others with reference to the dicarboxylic acids, aldehydes, transfusions of blood, injections of various tissue extracts, and we think also with reference to alcohol, the von Noorden oatmeal cure, and other diets which can cause edemas. Neubauer raised this point with reference to the glycerose experiments in 1910, also Edsall, and we have frequently reiterated the fact that the results of urine analysis even when taken in conjunction with blood analyses are not necessarily a true reflection of the metabolic status.

[8] Parnas, J., *Zentr. Physiol.*, 1912, xxvi, 671.

[9] Smedley, I., *Jour. Physiol.*, 1912–13, xliv, 203.

formation of glycogen. Since under the conditions of these experiments there would necessarily have been a lack of oxygen, her results are in harmony with those of Parnas. Later Embden[10] and his coworkers, for the first time employing a pure although uncrystallized *dl*-glyceric aldehyde prepared by Wohl's synthesis, studied its behavior in surviving tissues (liver and washed blood corpuscles apparently under conditions of relative asphyxia) with particular reference to its convertibility into lactic acid. It was found convertible into lactic acid under these conditions, and on the basis of these experiments Embden has constructed a scheme of sugar metabolism in which he makes a cleavage of the glucose molecule into optically active glyceric aldehyde a major step in the normal glucose breakdown.

In summarizing the experimental work up to the present time, the following facts appear to have been demonstrated: (1) Surviving tissues (with a deficient oxygen supply) cause the disappearance of *dl*-glyceric aldehyde added to or perfused through them, and at least a part of the triose is then converted into lactic acid. (2) The diabetic organism may convert *dl*-glyceric aldehyde into *d*-glucose. (3) Surviving livers perfused with oxygenated solution of *dl*-glyceric aldehyde may convert a part of the latter into glycogen. (4) When administered to normal animals and men, considerable amounts of glyceric aldehyde disappear and are probably utilized.

New Experiments with dl-Glyceric Aldehyde.

Material.—As a basis for these experiments we have used crystalline *dl*-glyceric aldehyde prepared by E. J. Witzemann from glyceric aldehyde diethyl acetal, which was obtained either by means of Wohl's synthesis, or more directly from glycerol oxidation mixtures as described in the two papers quoted above.[3] The material is a stable crystalline powder which is non-hygroscopic and dissolves slowly in water. It represents dimolecular crystals and is tasteless. It is also odorless, but after standing for days in a stoppered bottle a faint flavor of burnt sugar is noticeable. The crystals melt at 142°C., or 4° higher than any

[10] Embden, G., Kalberlah, F., and Engel, H., *Biochem. Z.*, 1912, xlv, 45. Kraske, B., 81. Kondo, K., 88. Von Noorden, K., jun., 94. Embden, G., Baldes, K., and Schmitz, E., 108. Embden, G., and Oppenheimer, M., 186.

previously recorded preparation. (138°, Wohl and Neuberg.[11]) Aqueous solutions of this material are clear and colorless, and unlike the dimolecular crystals, have a mild sweet taste. They reduce Fehling's solution in 1 to 5 minutes at room temperature, give a vivid aldehyde reaction with Schiff's reagent, and show all the characteristic reactions which have been described for *dl*-glyceric aldehyde. Observations made of its reducing power show that *dl*-glyceric aldehyde corresponds gram for gram with glucose. For the *detection of glyceric aldehyde in a body fluid* use has been made of the following properties: (1) Its power to reduce Fehling's solution at room temperature within 3 minutes or immediately at 50°C. (2) Its power to combine with phenylhydrazine acetate to yield insoluble crystals of glyceric aldehyde phenylosazone (m. p. 132°C.) slowly at room temperature, more rapidly at 50°C. (3) Its ability even in minute traces to produce a slowly deepening, finally vivid violet coloration when added to a solution of fuchsine decolorized with H_2SO_3 and freed of excess of the acid (Schiff's aldehyde test).

Toxicity and Tolerance.

Tests were made with rabbits and guinea pigs. The triose was administered in 2 to 7 per cent aqueous solutions subcutaneously and into the stomach. *When given by the stomach*, doses up to 2.8 gm. per kilo of body weight caused no apparent symptoms or qualitative urinary changes, although there was some diminution of the flow. A dose of 5 gm. per kilo (a rabbit of 1,400 gm. receiving 100 cc. of 7 per cent solution by stomach tube) was followed immediately by *diarrhea with free glyceric aldehyde in the passages*, a marked diminution in the rate of urination, *but no melituria. Thereafter for 10 days the urine contained albumin.* A dose of 6.8 gm. per kilo caused death in a guinea pig within 4 hours. There was no catharsis and no melituria was demonstrated. The animal died quietly, and at autopsy the right heart was filled with blood, the abdominal viscera showing passive congestion with only slight parenchymatous swelling and cloudiness, most evident in the kidneys. *Subcutaneously*, doses of 2.2 and 2.4 gm. per kilo given respectively to a rabbit and a guinea pig

[11] Wohl and Neuberg, *Ber. chem. Ges.*, 1900, xxxiii, 3095.

caused complete suppressions of urine and death, with no catharsis, and with autopsy findings similar to those mentioned above. Doses of 1 gm. per kilo were borne with no apparent symptoms.

To sum up: The average lethal dose by the subcutaneous route appears to have been about 2.2 gm. per kilo; by the alimentary route between 5 and 6 gm. per kilo. After as much as 1 gm. per kilo subcutaneously and 3 gm. per kilo by stomach no unfavorable symptoms were noted. Parallel experiments in the same set of animals showed for *d*-glucose given by either route a minimum lethal dose of 18 gm. per kilo, indicating a toxicity for this preparation of *dl*-glyceric aldehyde about three times that of glucose by stomach and eight times as great subcutaneously. Glyceric aldehyde was not found in the urine after oral or subcutaneous doses.[12]

Behavior of dl-Glyceric Aldehyde in Phlorhizin Diabetes.

Dogs weighing 10 to 15 kilos were given 1 gm. of phlorhizin (Merck) triturated in a mortar with about 20 cc. of olive oil once every 24 hours. Deglycogenation was effected by 3 hourly subcutaneous doses of (1: 1,000) adrenalin solution (Parke, Davis and Co.). Some of the experiments antedate the establishment of the routine which was later adopted, so that sometimes more and sometimes less than the usual 0.04 mg. of epinephrin per kilo was given, but the protocols are reproduced in sufficient detail to prove that deglycogenation was in every case satisfactory. All details as to the collections of urine and analytical methods were as described in the paper on narcotics.[2] Attempts were first made to administer the material by mouth. But although the diabetic dogs drank solutions spontaneously, the material was twice vomited, and owing to the cost of the preparation we did not risk a further loss. Subsequently it was given subcutaneously or by vein.[13]

[12] For the behavior of glyceric aldehyde when given by vein, see the following article.

[13] Dakin, H. D. (*J. Biol. Chem.*, 1913, xiv, 328), has warned against intravenous administrations on the ground that they may cause extra sugar to appear from sources other than the substance injected. This was apparently based on observations upon intravenous administrations in dogs prepared by simple phlorhizination with no supplemental deglyco-

The experiments with phlorhizinized dogs fall into two groups, A and B.

In Group A (Experiments I, II, III, and IV) glyceric aldehyde was given in 10 to 15 gm. single or subdivided doses with no supplemental treatment, to dogs weighing 13 to 17 kilos. All the animals died within 18 hours, death being preceded by marked urinary retentions which became noticeable within the first 6 hours but were more marked in the after-periods. In two experiments (I and II) no extra sugar appeared. In two (III and IV) the extra sugar was 4.49 and 3.95 gm., with gross increases of 2.46 and 2.63 gm. respectively. Thus in two of four experiments there were absolute and relative glucose increases corresponding respectively to 30 and 40 per cent of the weight of triose administered. These dogs had received epinephrin until it could be stopped for 6 hours and again given in the next 6 hours without changing the G: N ratio, so that the extra sugar which appeared could not have been derived from glycogen. It is accordingly attributed to the glyceric aldehyde, but it was not sufficient in amount to indicate a quantitative conversion. Reference to the protocol of Experiment IV will show that in the first after-period extra sugar reckoned in the usual way was 2.77 gm. But in this period there were marked absolute decreases in all the secretions, indicative of disturbed elimination, and under these conditions it is unsafe to interpret a rising G: N ratio as evidence of gluconeogenesis, especially when delayed.

In Group B (Experiments V, VI, and VII) the dosage of glyceric aldehyde was reduced to 5 gm., to lessen the toxic effects and urinary retentions. These dogs also received supplemental treatments consisting of intravenous injections of an alkaline diuretic salt solution. All the animals lived. In every case there was a marked gross increase of the urinary glucose and, except in Experiment VII, a corresponding output of extra sugar. The extra sugar output, following 5 gm. doses of glyceric aldehyde, were in Experiment V, 4.92 gm. (certified by a gross increase of 5.21 gm.), and in Experiment VI, 4.84 gm. (with a gross increase of 4.85 gm.). Thus the 10 gm. of glyceric aldehyde

genizing treatment as noted in our paper on narcotics.[2] But numerous control experiments convince us that there is no objection to this method if the dog is strictly glycogen-free.

TABLE III.

Experiments I, II, III, and IV. dl-Glyceric Aldehyde, Designated in Text as Group A, with No Alkaline Diuretic Treatment. Suppressions Marked in All. Definite Evidences of New Sugar in Only Two, and Moderate in These (III, IV).

Experiment No.	6 hr. period.	Glucose.	Nitrogen.	G:N	Extra sugar.†	Gross‡ sugar increase.	Acetone.*	β-Hydroxybutyric acid.	Total** acetone bodies.	Remarks.
		gm.	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
I	1–9									Animal received ostensibly 0.1 mg. epinephrin per kilo of body weight subcutaneously throughout the experiment. Unduly long preparatory period of 72 hours, owing to deterioration of the epinephrin solution, the real dosage being much smaller than intended.
	10	24.94	3.39	7.36						
	11	14.44	3.24	4.44						
	12	14.81	4.10	3.61						
	13	13.38	4.35	3.07						
	14	12.31	4.10	3.00						
	15	**11.50**	**3.61**	**3.18**	**0.0**	**0.0**				Glyceric aldehyde 10 gm. in 133 cc. H_2O subcutaneously in Period 15 followed by retentions. Death in Period 17.
	16	1.95	0.70	2.80						
	17									
II	1–3									Epinephrin 0.1 mg. per kilo of body weight every 3 hours to end of Period 5. Deglycogenation complete in 18 hours but this dosage is unduly severe.
	4	6.19	2.16	2.86						
	5	7.56	2.77	2.73						
	6	**7.06**	**2.63**	**2.68**	**0.0**	**0.0**				Glyceric aldehyde 10 gm. in 500 cc. H_2O subcutaneously at the rate of about 42 cc. every 30 minutes in Period 6.
	7	4.53	1.26	3.60	1.13	0.0				
	8	1.15	0.39	2.95						Retentions. Death in Period 9.
	9									
III	1–4									Epinephrin 0.033 mg. per kilo, every 3 hours to Period 12. G:N virtually constant after 36 hours.
	5–6	13.75	4.19	3.28						
	7–8	12.95	4.03	3.21						
	9–10	13.78	4.58	3.00						
	11–12	**12.28**	**4.19**	**2.93**			**0.428**	**0.289**	**1.056**	
	13	11.72	3.76	3.11						Epinephrin omitted in Period 13.
	14	11.41	3.91	2.92			0.391	0.340	1.041	Epinephrin resumed in Period 14. Dog glycogen-free.
	15	**14.03**	**3.17**	**4.42**	**4.49**	**2.46**	**0.212**	**0.293**	**0.673**	Glyceric aldehyde 15 gm. in 200 cc. H_2O subcutaneously in Period 15. Death in Period 16.
	16									

TABLE III—*Continued.*

Experiment No.	6 hr. period.	Glucose.	Nitrogen.	G: N	Extra sugar.†	Gross‡ sugar increase.	Acetone.*	β-Hydroxybutyric acid.	Total** acetone bodies.	Remarks.
		gm.	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
IV	1-4									Epinephrin ostensibly 0.1 mg. per kilo every 3 hours until end of Period 7.
	5	12.60	4.23	2.97						
	6	12.39	4.12	3.01						Deglycogenation in 18 hours with no injury, but the adrenalin had lost strength, and the actual dose was smaller.
	7	11.31	3.66	3.08			0.077	0.309	0.446	
	8	11.88	3.89	3.06			0.067	0.370	0.490	No epinephrin.
	9	10.06	3.28	3.06			0.056	0.296	0.396	Epinephrin resumed. Dog proved glycogen-free.
	10	**13.60**	**3.16**	**4.30**	**3.95**	**2.63**	**0.025**	**0.237**	**0.281**	Glyceric aldehyde 10 gm. subcutaneously. Epinephrin continued.
	11	7.70	1.61	4.08	2.77	0.0				Epinephrin as before.
	12									Death.

† Extra sugar = G − (N × G: N), when G = gm. glucose for a period, N = gm. nitrogen for the same period, and G: N = the average ratio for the preceding 12 hours.

‡ Gross sugar increase reckoned as gm. glucose in a given period minus the average gm. glucose for 12 hours preceding.

* Inclusive of acetoacetic acid.

** Reckoned in terms of β-hydroxybutyric acid.

given in Experiments V and VI gave rise to an output of 9.76 gm. of extra sugar, with a gross sugar increase of 10.06 gm., which clearly demonstrates a practically complete conversion into glucose of all the glyceric aldehyde administered. In Experiment VII extra sugar was only 1.46 gm.

In all the experiments of Group B evidences of retention were missing or moderate. Even in the earlier experiments, I to IV, they had been less marked in the experimental periods than subsequently, but in Experiments V to VII they were absent in the experiment periods. This difference is attributed to the more moderate dosage of glyceric aldehyde and to the treatment given. That the decreased dosage was not alone responsible for the difference and that the treatment also played an important part is demonstrated by the fact that in Experiments V and VI treatment was discontinued in the after-periods and in these periods signs of retention supervened, although less markedly than in the

experiments of Group A, whereas in Experiment VII, the alkaline diuretic treatment was continued into the after-periods, with the result that no retentions occurred at all. On the contrary, in this case both the glucose and nitrogen at the end of 18 hours were actually higher than they had been during the whole experiment, an occurrence which distinguishes this from all the

TABLE IV.

Experiments V, VI, and VII. dl-Glyceric Aldehyde. Experiments Designated in Text as Group B, with Alkaline Diuretic Treatment. Suppressions Moderate in Two, Negative in One. Gluconeogenesis Definite in All and Virtually Complete in Two (V, VI).

Experiment No.	6 hr. period.	Glucose.	Nitrogen.	G : N	Extra sugar.	Gross sugar increase.	Remarks.
		gm.	*gm.*		*gm.*	*gm.*	
V	1–4						Epinephrin 0.025 mg. per kilo of body weight subcutaneously every 3 hours.
	5	18.38	4.05	4.53			G : N still high after 30 hours, owing to small dosage.
	6						Epinephrin continued and analyses omitted.
	7	13.75	4.84	2.84			Epinephrin continued; low ratio and apparent deglycogenation after 36 hours.
	8	13.25	4.40	3.01			Epinephrin omitted, with no significant change in figures.
	9	15.00	4.47	3.35	1.90	1.50	Epinephrin resumed; shows trace of glycogen was present.
	10	**11.75**	**4.41**	**2.66**			No epinephrin. Alkaline diuretic (Fischer's solution) 20 cc. by vein.
	11	12.13	4.20	2.89			No treatment.
	12	**15.78**	**3.29**	**4.03**	**4.92**	**4.84**	Glyceric aldehyde 5 gm. (5 per cent solution), also 20 cc. Fischer's solution by vein.
	13	9.56	3.74	2.55			No treatment.
	14	9.25	3.61	2.56			No treatment. Dog strong and later recovered.

TABLE IV—*Continued.*

Experiment No.	6 hr. period.	Glucose.	Nitrogen.	G : N	Extra sugar.	Gross sugar increase.	Remarks.
		gm.	*gm.*		*gm.*	*gm.*	
VI	1–4						Epinephrin 0.025 mg. per kilo of body weight subcutaneously every 3 hours.
	5	12.63	3.05	4.14			Same. Ratio still high owing to small epinephrin dosage.
	6	11.69	3.58	3.26			Epinephrin continued.
	7	11.87	3.86	3.08			Epinephrin continued.
	8	10.50	3.61	2.90			No epinephrin.
	9	12.19	3.95	3.10	1.38	1.51	Epinephrin resumed and shows only trace of glycogen.
	10	**11.81**	**4.25**	**2.77**			Fischer's solution 20 cc. by vein increases nitrogen but not sugar.
	11	11.06	3.86	2.84			No Fischer's solution. No epinephrin.
	12	**11.13**	**4.13**	**2.70**			Fischer's solution 20 cc. by vein increases nitrogen but not sugar.
	13	11.56	3.92	2.94			No treatment.
	14	**16.56**	**4.17**	**3.97**	**4.84**	**5.21**	Glyceric aldehyde 5 gm. (in 5 per cent solution) subcutaneously, 20 cc. Fischer's solution by vein.
	15	9.75	2.62	3.72			No treatment. Retentions moderate. Dog recovered.
VII	1–4						0.03 mg. epinephrin per kilo of body weight every 3 hours.
	5	17.06	4.41	3.86			Epinephrin continued.
	6	15.00	4.27	3.51			No epinephrin.
	7	18.69	5.52	3.38	0.0	2.66	Epinephrin resumed; deglycogenation complete.
	8	12.63	3.40	3.71			
	9	14.06	4.27	3.29	0.0		Fischer's solution 31 cc. by vein.
	10	**16.88**	**4.43**	**3.81**	**2.31***	**3.53**	Glyceric aldehyde 5 gm. (5 per cent solution), also 31 cc. Fischer's solution by vein.
	11	13.31	4.47	2.98			Fischer's solution 31 cc. by vein. Note absence of retentions.
	12	17.94	5.66	3.17			Fischer's solution 31 cc. by vein. Blood in urine. Good recovery.

* Extra sugar reckoned on basis that G : N = 3.29, the ratio found in Period 9 with alkaline diuretic.

other six experiments and from the experiments performed with glyceric aldehyde in normal animals.

The diuretic alkaline solution selected consisted of 0.3 per cent Na_2CO_3 in 1.4 per cent NaCl solution, described by Fischer[14] for the clinical treatment of anurias, oligurias, edemas, etc. Other solutions could have been selected and might prove preferable for carrying out the principles of mild alkalinization and diuresis. But the Na_2CO_3—NaCl solution was chosen as containing no organic substance which could possibly be regarded as gluconeogenetic and because we had previously used it successfully for similar purposes. It was injected into the vein slowly in doses of 20 to 30 cc. once in the 6 hour period. In all experiments one or more of the fore-periods were utilized as controls. In Experiment V, Period 10, the dog received 20 cc. of Fischer's solution by vein with no appreciable effect. There was indeed in this period some *decrease* of glucose below the figures of the preceding period; but this was to have been expected even if no therapy had been given, since in the preceding period epinephrin had been administered and, the animal having evidently retained a trace of glycogen, the urine for this period contained extra sugar. In Experiment VI, Period 10, Fischer's solution caused an increased output of nitrogen, but did not affect the glucose. It was given again in Period 12 with similar results. In Experiment VII, Period 9, it was given again and here caused a marked increase in nitrogen with a simultaneous rise in glucose, but nothing which could be mistaken for extra sugar since G: N was somewhat decreased. It was evident from these controls that the intravenous administration of Fischer's solution could not in itself cause any extra sugar to appear in the urine.

Having controlled in each experiment the effects of the alkaline diuretic solution alone, a similar dose was given after the administration of the glyceric aldehyde in each experimental period proper, with the results noted. In Experiment VII the solution was given in the fore-period, the experimental period, and in the first and second after-periods. The results were that the nitrogen showed a progressive increase to the end of the experiment with no such decrease in the glucose as char-

[14] Fischer, M. H., Oedema and Nephritis, New York, 2nd edition, 1915. 549.

acterized the after-periods of all other experiments. The urine of the last period, 17, contained blood which further abnormally increased the nitrogen. The animal recovered rapidly.

DISCUSSION.

Our chief interest in glyceric aldehyde lay in the fact that it might be regarded as glucose, split or dissociated but not otherwise altered, and that it might afford an available substance with which to test the theory that the diabetic defect consists in a decreased power of the tissues to dissociate glucose and perhaps allied substances. The remarkable ease with which glyceric aldehyde oxidizes and polymerizes outside the body in the presence of even small traces of alkali suggests that it is naturally more highly dissociated and might be more readily attacked in the diabetic body than glucose, as Neuberg stated to be the case, but did not prove. If it were more readily utilized in the body, the fact would have theoretical and clinical significance. The experiments show that although pure glyceric aldehyde is not wholly non-toxic, nevertheless it is borne without apparent injury by normal animals in single doses which would correspond to 150 or 200 gm. by mouth for a man of average size, so that its use as a food would not be impossible. When given to the diabetic it is capable, however, of becoming wholly transformed into glucose, and this transformation would appear to preclude the possibility of its having a greater utilization rate in the diabetic organism than glucose itself. It falls in this respect in the same category with glycerol, lactic acid, levulose, and several other substances which in the past have for unsound reasons been recommended in the treatment of diabetes.

The single fact that glyceric aldehyde may be converted quantitatively into glucose when it is introduced under the conditions of these experiments into the living body in a state of total diabetes would not finally settle the point of theoretical interest as to whether it might not be utilized in isolated diabetic tissues under other conditions, but certainly a mere failure to find in the urine of a diabetic individual the glucose equivalent of the glyceric aldehyde given does not justify the conclusion that the missing part has been utilized.

SUMMARY.

Chemically pure *dl*-glyceric aldehyde may be ingested by normal animals (rabbits and guinea pigs) in single doses of 1 to 2 gm. per kilo of body weight without noticeable symptoms, except diminution of urine. The same applies to subcutaneous doses of 0.3 to 1 gm. per kilo. A dose of 5 gm. per kilo by stomach was toxic but not fatal in a rabbit (diarrhea with unchanged glyceric aldehyde in passages, oliguria, albuminuria). A dose of 6.8 gm. by stomach killed a guinea pig. By the subcutaneous route, guinea pigs were killed with doses of 2.2 to 2.4 gm. per kilo, death being preceded by anuria. Autopsies showed slight passive congestion and parenchymatous changes in the abdominal viscera, especially the kidneys. No glyceric aldehyde was detected in the urine when urine was secreted. In completely phlorhizinized dogs and in human diabetes glyceric aldehyde may be converted into glucose. A virtually complete conversion is demonstrated if suitable precautions are taken to avoid toxic retentions due to the effects of glyceric aldehyde on the kidneys and tissues generally (small doses of glyceric aldehyde, alkaline diuretic therapy).

Concerning the technique of metabolism experiments with phlorhizinized dogs, it has been noted (*a*) that three hourly subcutaneous doses of 0.04 mg. of epinephrin per kilo of body weight generally serve to deglycogenize the animal within 24 hours without shortening the experiments or introducing undesirable effects; and (*b*) that by treating phlorhizinized dogs with a suitable alkaline diuretic solution to combat the diabetic acidosis and the added toxic effects of the experimental substance, the passage of metabolites into the urine may be facilitated.

STUDIES ON THE THEORY OF DIABETES.

VII. THE INTRAVENOUS TOLERANCE LIMIT FOR *dl*-GLYCERIC ALDEHYDE AND THE IMPROBABILITY THAT IT IS A CHIEF INTERMEDIATE IN GLUCOSE CATABOLISM.

By W. D. SANSUM and R. T. WOODYATT.

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(Received for publication, December 9, 1915.)

We[1] have made a preliminary report concerning a quantitative pump devised for the purpose of making intravenous injections at exact rates for long periods of time, and of experiments in which different sugars have been injected at exact rates in different normal species and in certain pathological conditions in man. A detailed report is in the course of preparation. Certain results are of interest in connection with the preceding report on glyceric aldehyde.

By timed intravenous injections it has been found that pure *d*-glucose may be introduced into the peripheral venous blood of normal resting dogs, rabbits, and men at uniform rates below 0.8 gm. per kilo of body weight hourly for from 2 to 12 hours and presumably longer, without causing glycosuria. But when the rate of injection is increased to between 0.8 and 0.9 gm. of glucose per kilo per hour, glycosuria begins. The tolerance limit for glucose given by peripheral vein appears to be very close to 0.85 gm. per kilo of body weight per hour. This applies to glucose given in gram molecular (or 18 per cent) solution, and also to less concentrated solutions, provided that there is sufficient salt to maintain a suitable tonicity.

Pure crystalline *dl*-glyceric aldehyde, the same preparation as that used in the experiments described in the preceding article, has been injected into the ear veins of rabbits in 9 per cent aqueous

[1] Woodyatt, R. T., Sansum, W. D., and Wilder, R. M., *J. Am. Med. Assn.*, 1915, lxv, 2067.

solution and in 2 per cent solution in 1.4 per cent salt solution, at exact rates, and it has been found that unchanged glyceric aldehyde begins to appear in the urine when the rate of injection exceeds 0.1 gm. of glyceric aldehyde per kilo of body weight hourly. The normal tolerance limit for *dl*-glyceric aldehyde when injected into the peripheral venous blood of rabbits lies close to 0.15 gm. per kilo per hour. The *dl*-glyceric aldehyde in the urine was detected by the power of the urine to reduce Fehling's solutions visibly within 1 minute at room temperature, and very markedly in 3 to 5 minutes, by its power to produce the characteristic slowly developing violet color with Schiff's decolorized fuchsine test for aldehydes, and by the absence of any optical variation in the urine which gave a zero reading in the polariscope before and after the appearance of reducing substance in it. It is clear from these experiments that the tolerance limit for intravenously administered *dl*-glyceric aldehyde (0.15 gm. per kilo per hour) is about $\frac{1}{6}$ of that for glucose (0.85 gm. per kilo per hour). This does not imply, of course, that the body cannot utilize more than 0.15 gm. of glyceric aldehyde per kilo per hour if the rate of the injection into the peripheral vein is accelerated, but apparently it cannot use more without an overflow of glyceric aldehyde into the urine unless the urine is suppressed.

We have given *d*-glucose by peripheral vein to dogs for periods of 8 hours at the rate of 5.4 gm. per kilo per hour, without seriously impairing health, and at this rate of injection after the first 4 hours close to 2.7 gm. per kilo, or 50 per cent of the glucose given may be steadily excreted hourly, leaving 2.7 gm. per kilo hourly which continuously and permanently disappears. Most or all of this 2.7 gm. per hour must be utilized.[2]

If all the 2.7 gm. of glucose utilized were first split into glyceric aldehyde, then in these experiments we would in effect be introducing into the body 2.7 gm. of glyceric aldehyde per kilo per hour. But by experiment it has been found that this would be

[2] If this were not the case, we should expect the physiological effects of accumulations of unburned glucose in the tissues; *viz.*, fall in the urinary output, thirst, fever, chills, and convulsions, which are seen when the rate of injection of glucose exceeds a certain value; or, avoiding dehydration by giving larger volumes of water, heart failure, and water logging. These effects were absent.

lethal, whereas the dogs which received 5.4 gm. of glucose per kilo per hour for 8 hours showed no serious effects. Also an injection rate of only 0.15 gm. of glyceric aldehyde per kilo per hour produces a definite triosuria, so that if even 5.5 per cent of the 2.7 gm. of glucose were converted into glyceric aldehyde we should expect to find easily demonstrable quantities of glyceric aldehyde in the urine, which we do not find. Even when giving 7.2 gm. of glucose per kilo per hour we have not succeeded in demonstrating glyceric aldehyde in the urine. Therefore it would seem impossible that more than 5 per cent at most of all the glucose which is utilized in the normal body is split into glyceric aldehyde.

The question arises as to what percentage of the 2.7 gm of glucose—unaccounted for in such an experiment—is actually oxidized, and how much polymerized or even reduced. Through the courtesy of Dr. Harvey Cushing, respiration experiments bearing on this question have been made possible. They were carried out by Dr. Walter M. Boothby and Miss Irene Sandiford of the Respiration Laboratory in the Peter Bent Brigham Hospital, Boston, the writers cooperating. The full report of this work will form a separate publication, but with the consent of the other laboratory, we may state that in one experiment a dog weighing 19.6 kilos received glucose steadily by vein for 13 hours at the rate of 3.522 gm. per kilo per hour and that after an equilibrium was established, the urinary excretion rate averaged 1.141 gm. per kilo an hour, leaving 2.381 gm. per kilo which disappeared hourly. The respiratory data obtained by Boothby and Sandiford would indicate that, roughly, 25 per cent of this 2.381 gm. was oxidized. In this case then the rate of glucose oxidation was approximately 0.6 gm. per kilo per hour. If only 25 per cent of this passed through a glyceric aldehyde phase, it would suffice to cause a triosuria. This rate of glucose oxidation can doubtless be greatly exceeded by employing higher injection rates and exercise, but the data given suffice to show that even in the oxidative utilization of glucose as distinguished from other chemical fates the change of glucose into two molecules of glyceric aldehyde can scarcely constitute a major step.

So far as we know, there are no experimental data which prove

that any glyceric aldehyde is formed in the breakdown of glucose in the body. But if there is, it represents a minor fraction. According to the theory under discussion in these papers, glucose exists in the body as saturated molecules in a state of dynamic chemical equilibrium with the ions or unsaturated residues into which it is dissociated. Among the dissociation fragments which participate in the glucose equilibrium, there could be some which might be designated as ions of glyceric aldehyde; *e.g.*, $CH_2OH - CHO - \overline{COH} + H^+$, or as methylene derivatives of glyceric aldehyde, such as $CH_2OH - \underset{\wedge}{C} - COH + HOH$ or their equivalents. If glyceric aldehyde were introduced into the body it would by dissociating yield residues identical with these, so that glyceric aldehyde would be capable of entering into the glucose equilibrium, as noted in the preceding article. But it would not follow that any measurable concentration of molecular glyceric aldehyde should necessarily exist as such in the living body.

Many writers have regarded glyceric aldehyde as an important intermediate in the breakdown of glucose in the body. In the scheme of glucose catabolism constructed by Embden, and much quoted by others, the main path in the catabolism of glucose is depicted as an aldol depolymerization into optically active glyceric aldehyde which then changes into lactic acid. We believe that this particular change of glucose into glyceric aldehyde does not constitute an important step in its disintegration in the living body and that conceptions of the breakdown of glucose in which the process is depicted as though it proceeded from one fixed molecule to another are fundamentally inadequate.

EXPERIMENTAL STUDIES ON GROWTH.

I. METHODS.

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(Received for publication, January 28, 1916.)

The series of articles of which this is the first will describe the results of experimental researches on the influence of certain dietary factors upon the process of growth and phenomena incident thereto in white mice. To accomplish the purpose of these researches it is imperative to exclude disturbances of growth due to factors, such as infections or exposure, other than those which the researches are designed to investigate. Accordingly before undertaking the experiments which will be described in subsequent communications of this series we devoted considerable attention and some preliminary experimental work to the construction of an environment for our experimental animals which would safeguard them as far as possible from zymotic diseases and enable us readily to prevent the spread of such diseases among them by infection from the sporadic cases which are practically impossible to avoid. It was also essential that the environment should be such as to exclude, as far as could be foreseen, all other variable factors which might conceivably exert an appreciable effect upon the growth of the animals. The methods which we have contrived to meet these desiderata have proved highly satisfactory, inasmuch as we have been able to maintain over 75 per cent of our control animals in healthy condition to the age, at the date of writing, of nearly 18 months, the great majority of deaths among adult animals having been due either to injuries attributable to the belligerent habits of the males or to the spontaneous incidence of tumors. Moreover, the methods are very convenient, permitting rapid manipulation, so that, when necessary, one individual with little assistance can maintain entire charge of about 500 animals. Since a descrip-

tion of the methods employed must in any case precede the description of the experimental results obtained by their aid we have thought it advisable to devote the first article of this series to this purpose.

The essential factors in the environment of an animal in captivity may be summarized under the following heads: (1) the construction of cages and nests; (2) the cleansing of the cages and nests and the removal of debris; (3) the methods of administering food and water; (4) the character of the food administered; (5) the condition of temperature, humidity, etc.; (6) the methods of combating the spread of infections; while in experiments upon growth involving the weekly or biweekly weighing of 400 to 500 animals, the methods employed in identifying and weighing the animals are also of considerable practical importance. These various factors will be dealt with *seriatim*.

Construction of the Cages and Nests.

The cages which we employ are constructed, with some modifications essential to our special purpose, from the design recommended by Daniel.[1] In this we have been greatly assisted by the advice of Dr. Daniel, to whom we desire to express our indebtedness.

Our cages differ from those illustrated by Daniel in the article to which reference has been made, in the following particulars. Since we did not administer milk to our animals the milk receptacle is eliminated. Since we employ a different device for administering grain the food funnel and food cup illustrated by Daniel are eliminated. The nest boxes occupying only about two-thirds of the width of each compartment, a narrow runway is left between the side of the nest and the partition of the adjacent compartment (or side of the cage) on the right, the water receptacle extending down into this runway so as to be conveniently reached by a mouse from the wire mesh floor of the compartment. This modification was introduced because it was found that if the opening of the water receptacle was directly over the nest box as in Daniel's design, water tended to collect on the roof and seep into the interior of the nest box; furthermore,

[1] Daniel, J. F., *Am. Naturalist*, 1912, xlvi, 591.

the surfaces of the nest boxes which we employ being slippery, if they had extended from side to side of the compartment the mice could not have climbed them to reach the water. Incidentally our arrangement afforded the mice more running-space and, since they utilized the water receptacle as a means of climbing up and down to and from the roof of their nest boxes, it afforded them a healthful degree of exercise. Finally, the back of the cage, as well as the front, sides, and partitions, consisted of window-glass of medium thickness fitting into grooves in the wooden frame, the galvanized iron back employed by Daniel being deemed undesirable on account of the possible chemical action of stale urine upon it, leading to the formation of metallic salts which might be absorbed by the animals and exert an unknown effect upon their growth and well-being. The danger of this in the case of the wire mesh floor was much less because moisture would tend to collect on the lower side of the mesh, remote from the animals, and incrustations would tend to be displaced by the movements of the animals and fall down to be collected and removed along with the other debris.

Fig. 1 shows our cage from the front, with nests (A), food tubes (B), water tubes (C), and receptacles (E and F), *in situ*. The glass sheets forming the sides and partitions of the cage are fitted into grooves cut in the upper part of the wooden frame, while the sheets of glass forming the front and back are held in place below the upper part of the frame by the sides and partitions which accurately fit the space between them. The top of the cage is formed, at the back, of a strip of wood $2\frac{1}{2}$ inches broad, perforated with holes for the water receptacles to pass through and provided with rests for the hinged lid $11\frac{1}{2}$ inches wide which is attached to it and forms the front part of the top of the cage. The whole of the top part of the cage is removable, being attached to the body of the cage by three hooks. The floor and the front or hinged portion of the top of the cage are covered with $\frac{1}{4}$ inch wire mesh, and the glass sides, front, back, and partitions are so arranged that at no part of the cage can the animals gain access to any wooden surface excepting when, as occasionally happens, they climb upside down on the lid of the cage. This arrangement prevents the wetting of wood by urine and the unhygienic conditions arising therefrom.

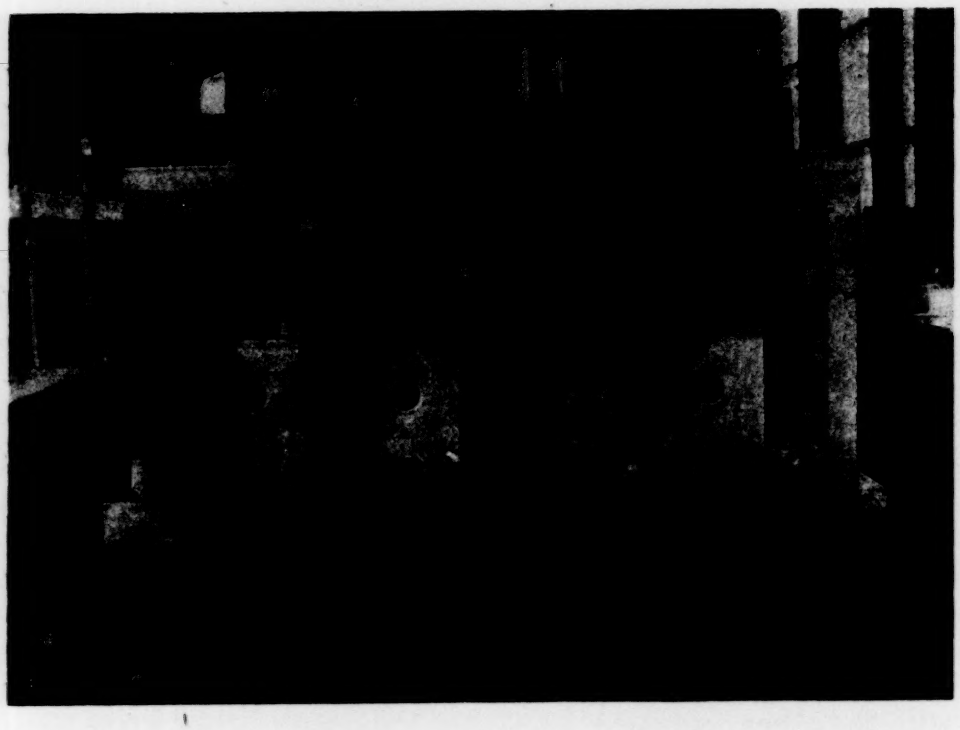

FIG. 1. Mouse cage with nests (A), food tubes (B), water tubes (C), waste grain tray (E), egg receptacles (F), and grain tube stoppers (D), *in situ*.

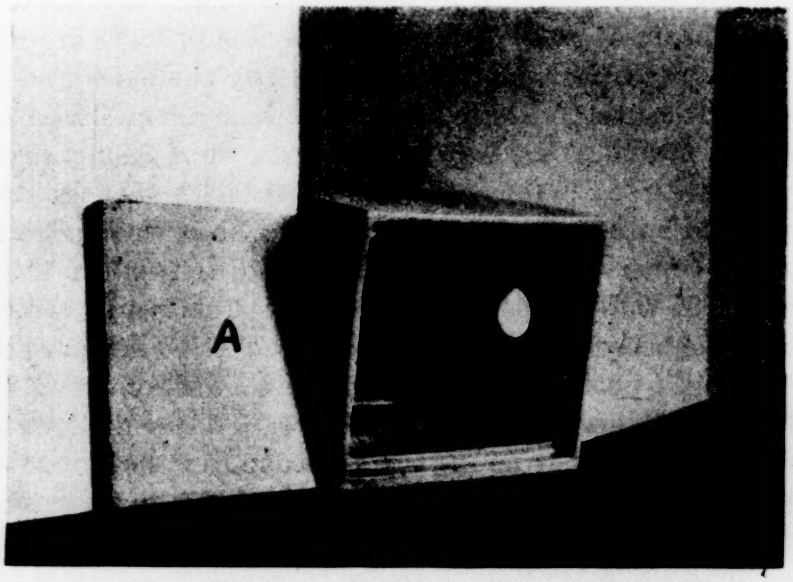

FIG. 2. Terra-cotta nest box. (A) removable side.

The dimensions of the cages are approximately as follows: length of top of cage $27\frac{1}{2}$ inches, width $13\frac{3}{4}$ inches, width from front to hinge $11\frac{1}{4}$ inches; length of base of cage $29\frac{1}{4}$ inches, width 15 inches; total inside length of cage $25\frac{3}{4}$ inches, width $11\frac{1}{2}$ inches.

The nests which we employ[2] are made of terra-cotta glazed on every surface which is accessible to the animals. Fig. 2 shows the general construction. They are $5\frac{3}{4}$ inches high, $6\frac{1}{4}$ inches from back to front, and $4\frac{3}{4}$ inches wide, all outside measurements, the walls being $\frac{5}{16}$ of an inch thick. The inside surface of the bottom of the nest is channeled and slopes towards the middle, where a small orifice discharges moisture. The entrance to the nest is circular, measuring $1\frac{1}{4}$ inches in diameter. The lid or loose wall A fits into the side of the nest where it rests against a rim which projects about $\frac{1}{4}$ inch from the inner walls of the nest on all four sides; the inner surface of this lid is glazed while the outer, which is pressed up against the left hand partition or side of the cage, is unglazed. This arrangement is preferable to having the loose wall of the nest forming the roof, as this would allow urine to seep in around the edges. Moreover, this arrangement permits one surface of the loose wall, namely, that which is inaccessible to the animals, to be unglazed, which fact facilitates the manufacture of the cages. The only other unglazed surface is the under surface of the base of the nest, a surface which is also, of course, inaccessible to the animals. We prefer terra-cotta nests to wooden or metal nests for a variety of reasons. Wooden nests tend to become sodden with urine and any waterproofing substance would be gnawed by the animals and might affect them deleteriously; moreover, wooden nests are not easily sterilized. Metal nests are subject to more sudden fluctuations of temperature and, moreover, we desired to avoid metal surfaces for reasons outlined above. The terra-cotta nests retain for a considerable time the warmth which the animals communicate to them, thus securing equable conditions of temperature within the nests, and, whenever it appears desirable, the entire nest can be plunged into mercuric chloride solution and then, after careful washing and drying, be employed again without fear of communicating infection by its use.

[2] The nests are manufactured for us by the Steiger Terra-Cotta Co. of San Francisco, at a cost of 60 cents each.

The small perforation seen in the photograph above the entrance to the nest was meant for a purpose which was abandoned early in the experiment and is unessential to the design of the nest.

The bedding employed was a vegetable fiber known to the local trade as "silk floss." It is used for upholstering and is probably identical with or similar to the fiber known elsewhere as "kapok," which is the floss or seed-hair of *Eriodendron anfractuosum*. This floss has the advantage of not matting so readily as absorbent cotton, and, moreover, it does not cling to the animals as absorbent cotton tends to do. Only in certain well defined pathological conditions was any tendency noticed for the floss to adhere to any part of the animal. The supply of floss in any one nest should not be too abundant, otherwise the animals take the initiative themselves and eject the excess from the nest. In hot weather they frequently draw the bedding out of the nests and sleep outside, replacing the bedding and retiring within the nests of their own accord as the temperature falls.

Removal of Debris and Cleansing of Cages.

The cage shown on the left hand side in Fig. 1 gives an idea of the arrangement of the cages. They were placed end to end and back to back in rows five cages long and two deep, on tables of which the edges are raised about $1\frac{1}{4}$ inches, thus forming a sort of tray. Along the entire length of the top of the table was laid a sheet of stout wrapping paper, and the ends of the cages were supported by narrow wooden slats laid across from edge to edge of the table at the requisite intervals. The debris from the cages thus fell through the wire mesh floor on to the wrapping paper. Once a week the cages were lifted down, and the debris was shaken into the middle of the paper which was then folded up and removed, following which a fresh sheet of paper was laid down and the cages were replaced. The removal of debris by this means was expeditious and thorough and this plan was preferred to the zinc lined tray flushed by water which is recommended by Daniel, first because it was found that the removal of debris by this means was not so rapid as that just described, and secondly because we feared the effect upon the well-being of the animals of the frequent presence of water underneath the cages.

The cages were cleansed once a week. This was readily accomplished by removing the top of the nest, washing the glass surfaces with a wet cloth, and subsequently drying them, and scrubbing the wire screen floor with a dry brush provided with stiff wire bristles. From time to time the cages were more thoroughly cleansed by removal of the glass sides, ends, and partitions, and thoroughly scrubbing the woodwork and wire screen with soap and water. The nest boxes and bedding were renewed once a week, and the used nests were thoroughly washed and dried before reemployment.

Nature of the Food and Method of Administering Food and Water.

The object of the experiments, except in certain instances to be specified later on, being to study the effect of abnormal additions to an abundant, normal, and well mixed diet, a wide variety of food was given to the animals and a constant supply of food was offered to them. The staple consisted of rolled barley which was supplied to them by means of the receptacle marked B in Fig. 1. This consisted of a heavy walled glass test-tube 9 inches long and $1\frac{1}{4}$ inches in diameter, a hole being blown in the lower end large enough to readily permit the egress of a single grain. Thus contamination of the grain by feces is avoided with the possible spread of infection involved therein, and, moreover, the animals are put to some trouble to get the grain and are not so wasteful of it as they are when it is supplied to them in open vessels. Even so, however, they frequently amused themselves by pulling out grains and throwing them down, with the result that they fell in large quantities through the wire screen uneaten. In order to minimize this waste the shallow glass trays E (Petri dishes) were introduced to catch this grain and keep it accessible to the mice for a brief period until consumed. These trays were kept in position under the opening of the grain tubes by means of a narrow strip of aluminum foil bent into a double hook, one arm of which was bent over the edge of the tray and the other under the adjacent partition. The tubes were suspended by a wire hook from the top of the partition. At first they were left open at the upper end, but it was found that the animals not infrequently climbed up to the top of the tube and attempted to reach the

grain from above, with the result that two or three fell head first into the tubes and were suffocated before they were discovered. Hence the grain tubes were closed at the top by the removable stopper D which consisted simply of a short length of glass tubing of about $\frac{1}{2}$ inch in diameter and 1 mm. in thickness of the wall, one end being sealed and then blown out into a bulb and the tube being then cut in such a way as to leave the bulb provided with a short neck which was inserted into the grain tube.

The grain tubes were replenished twice daily with the aid of a scoop and a metal funnel fitting into the top of the tube.

Another item of the dietary which is supplied daily (except Sundays) is the mixed white and yolk of eggs. The whites and yolks of eggs are beaten up together and strained, 5 cc. of the mixture being supplied to six mice (*i.e.*, to each compartment). The mice eat this greedily and it is usually completely consumed a few minutes after being offered to them. This was made the vehicle for administering the special substances the action of which upon growth we wished to determine. The egg was supplied in Syracuse watch glasses (F in Fig. 1) which were washed and renewed daily.

Fresh leaves of lettuce were supplied twice weekly, and on Sundays thoroughly dried bread. It is essential that bread supplied to mice should be thoroughly dry, otherwise intestinal disturbances make their appearance. Our procedure is to cut the bread into small cubes and dry thoroughly over a radiator. Only ordinary bakers' bread was employed, French and other fancy varieties being avoided.

The water is administered as in Daniel's cages in glass tubes sealed at the upper end, with the lower end melted down until only a small orifice remains. Instead, however, of holding them in place by rubber bands we found it more convenient to accomplish this by blowing a bulb at the sealed end, too large to pass through the orifice in the cover of the cage. The tubes employed are $\frac{1}{2}$ inch in diameter and $11\frac{1}{2}$ inches long and are made out of glass tubing of about 1 mm. in thickness of the wall. They are filled daily or every second day. From time to time they are cleaned out with the aid of a fine brush of the type generally listed in catalogues as "glass tube cleaners," cut in half and soldered on to a length of stiff steel wire. For filling the drinking

tubes we employ a large wash bottle provided with a rubber bulb (without a valve) attached to the air inlet tube of the bottle by a convenient length of rubber tubing. In this way pressure could be communicated by pressing the bulb, and it could be instantly reduced, if desired, by simply relaxing the bulb. The water exit tube is attached by means of a short piece of rubber tubing to a short length of tubing drawn out into a point which fits conveniently into the orifice of the drinking tubes.

The Conditions of Temperature, Humidity, etc.

The room in which the animals were kept was large and well ventilated and lighted, being supplied with a row of windows on two sides.

The source of heat was a single steam heated radiator provided with a valve permitting the occasional escape of small puffs of cooled steam. This, together with the constant ingress of fresh air (but not wind) kept the atmosphere humid but not unpleasantly so. At a late hour in the evening (about 10 p.m.) the steam pressure began to fall owing to the banking of the fires. Thereafter the room cooled down slowly until an early hour in the morning when the fires were renewed and the steam pressure rose again. On warm days the steam heat was turned off altogether, while on the other hand a small electric radiator was employed in exceptionally cold weather. The source of heat was in a corner of the room remote from the cages, so that the temperature conditions over the area occupied by the cages were tolerably uniform.

During 5 months, fairly representative of the entire period occupied by the experiments, the temperature of the room was registered by a self-recording thermometer. The mean temperature at midday during this period was 20.9°C. The mean diurnal maximum (from 4 to 6 p.m.) was 24.1°C., and the mean diurnal minimum (from 6 to 8 a.m.) was 17.5°C. The mean variation in temperature throughout the 24 hours was 6.6°C. The highest temperature recorded was 31.1°C., and the lowest, on one occasion only, owing to a failure of the steam heat, was 9.4°C.; with this exception the lowest temperature recorded was about 12°C.

The temperature was therefore subject to considerable fluctuation, a condition which, according to Chalmers Mitchell,[3] is conducive to the well-being of animals in captivity. Extreme variations, such as those found by Daniel to be deleterious, did not, of course, occur, the maximum variation noted in any 24 hours being 12°C.

Methods of Combating Infection.

White mice are fairly hardy animals, the only disease to which they are especially subject being β-paratyphoid.[4] This made its appearance in a certain number of animals about 4 or 5 weeks after mixing different litters;[5] *i.e.*, when the animals were from 8 to 9 weeks old. From 10 to 15 per cent of the animals then displayed symptoms of diarrhea and emaciation, passing blood-stained feces. These animals were promptly killed, the records of their weights discarded, and they were replaced by fresh animals, the infected cages, food and water receptacles, etc., being washed with alcohol. This stage past, hardly any further cases of paratyphoid were observed, and, save in one instance when a whole compartment (six) of adult animals suddenly developed symptoms, the cases observed were confined to a single mouse in a cage and led to no epidemic. The glass partitions naturally prevented the spread of infection from one compartment to the adjacent compartments.

Among adult animals sporadic cases of pneumonia occasionally occurred. Only in one instance when three animals in one compartment had developed the disease before it was detected did anything in the nature of an epidemic occur. The symptoms were emaciation and severe dyspnea. The animals were killed, or, in doubtful cases, isolated for further observation, and the infected nests and cages sterilized in the manner described above. Postmortem examination in these cases showed the lungs to be engorged and blood-red. Cancer of the lungs which occurs frequently among female mice resembled pneumonia in external

[3] Mitchell, P. C., *Proc. Zool. Soc., London*, 1911, 469.

[4] Infected animals were examined for us by Dr. K. F. Meyer, who identified the infection as β-paratyphoid.

[5] It was not so frequently observed when the litters were kept segregated.

symptoms, but the postmortem appearance of the lungs, of course, differed too greatly from that of pneumonia to permit any uncertainty as to the cause of death.

Infected wounds, due to injuries received in fighting, were observed occasionally among the males. If the infection was serious the animal was killed to avoid confusion of the records by the inclusion of possibly abnormal figures.

The animals were frequently examined for external parasites, which according to both Daniel and Chalmers Mitchell are deleterious to the well-being of animals in captivity. None were ever found. In one or two instances animals which came to postmortem had a small cyst on the surface of the liver containing a long worm coiled up in it. In each instance death could be attributed to another cause and the animals appeared to have been in no appreciable degree affected in their health by the presence of the parasite. With this small number of exceptions internal parasites were not observed in any of the animals which came to autopsy.

The weights of animals found suffering from infections, cancer, or dying from unknown causes, were deleted from the records for a period of 5 weeks preceding death. This was deemed ample allowance to exclude abnormalities in weight attributable to the diseased condition, for pneumonia develops rapidly, death occurring within 2 or 3 days after the first symptoms are observed. Paratyphoid produces very obvious emaciation and feebleness within a like period, and cancers which could be observed externally usually reached the stage of ulceration within 3 weeks after the first small lump was detected.

Methods of Identifying and Weighing the Animals.

The animals were identified by means of nicks and holes punched in the ears, a small punch such as is commonly employed by chicken breeders for marking their birds being used for this purpose. The different classes of experimental animals, *i.e.*, controls, pituitary-fed animals, etc., were distinguished by means of punch-holes in the ears, to guard against any possibility of accident to the cages leading to temporary mixing of the animals. This contingency never arose and the punch-holes were therefore never

required for purposes of identification. The animals, six in number and of the same sex, in each compartment were differentiated by means of nicks in the ears and distinguished in the records by numbers and the letter designating the compartment, the capital letter designating the experimental class, and the symbol designating sex. The woodwork of the cages containing different experimental classes was painted in different colors as a mnemonic guide.

The sexes were kept separate to avoid complication of the data by pregnancies.

The time consumed in the weekly or biweekly weighing of so many animals was a serious consideration. It was necessary to weigh to the desired degree of accuracy as rapidly as possible. Ordinary balances were too sensitive and their period of oscillation too long. Spring balances were tried and found insufficiently accurate. Finally we employed and found in the highest degree satisfactory a modified form of the triple beam balance.[6] The balance, as modified, is shown in Fig. 3. The modifications which we added consist of the brass cup C into which dips a disc-shaped plunger affixed by a length of stiff wire to the under side of the lower beam. The wire was cut to such a length that the disc could not touch the bottom of the cup nor emerge from the surface of the liquid paraffin ("medicinal petroleum") with which the cup was filled. This damped the oscillations of the balance, causing it to come almost instantly to equilibrium.

The threaded milled brass counterpoise B and low pitched screw-thread A which permit its accurate adjustment were attached to the front of the beam by means of brass posts screwed into the lower ends of the beam. This counterpoised the beaker in which the animals were placed to be weighed. The capacity of the balance, after counterpoising the beaker, is 111 gm., it reads to 0.1 gm., and is sensitive to 1 or 2 cg. Animals of 4 weeks of age or over were weighed to the nearest 0.5 gm., the daily variation equalling or exceeding this amount. Younger animals were weighed to the nearest 0.1 gm.

With the aid of this balance, which with suitable modifications of counterpoise and capacity could be readily adapted for weigh-

[6] This balance is illustrated under No. 330 in Catalogue C issued by Eimer and Amend, New York, 1913.

ing other small animals, the time consumed in weighings is reduced to a minimum, it being possible without undue haste, with clerical assistance in taking down the observed weights, to weigh no fewer than sixty animals in an hour, the time consumed in removing the animals from their compartments, renewing the nest box,

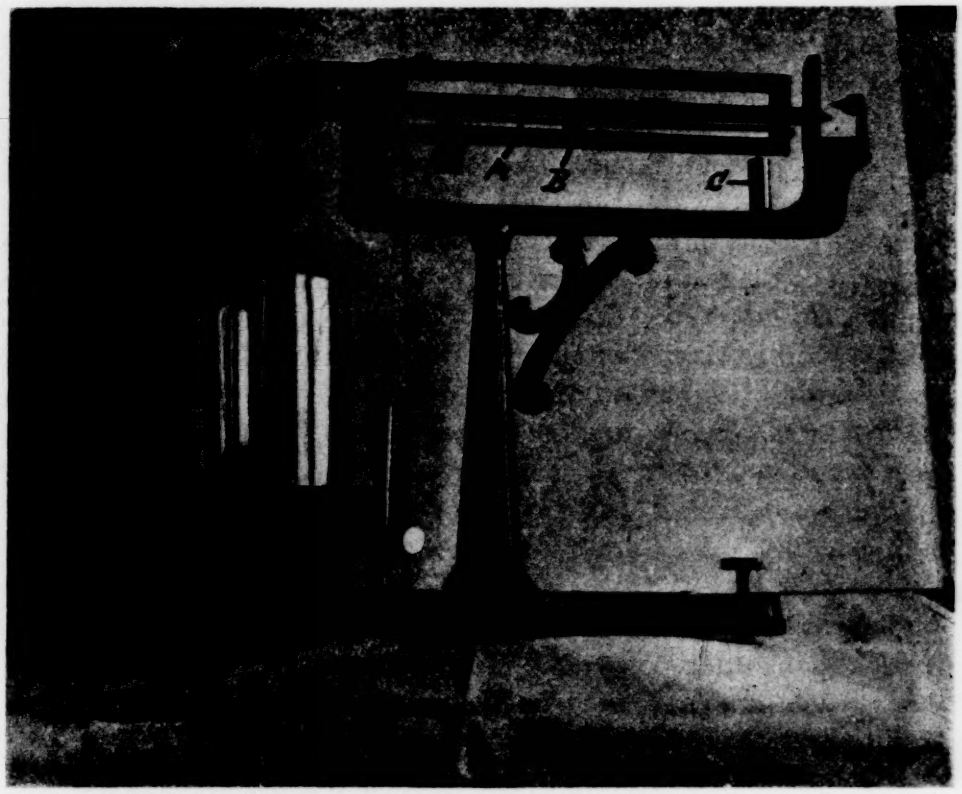

FIG. 3. Balance employed in weighing mice, showing screw-thread (A), and milled brass counterpoise (B). A plunger attached to the lower end of the beam dips into liquid paraffin contained in the brass cup (C).

and replacing the animals when weighed being included in this estimate.

We have weighed mice at short intervals throughout the day from 8 a.m. to 10 p.m., and in this way have found that their weight is subject to fluctuations occurring at fairly definite times in the day, dependent upon the feeding habits of the animals. A favorite time for feeding would appear to be the early evening, the weight always being high at about 10 p.m. In the early

morning the weight is again low and rises during the morning to fall again during the afternoon. We therefore always weighed the animals at the same time; namely, the middle of the afternoon, when the amount of material contained in the alimentary canal is relatively small.

Sources of Error in the Weighings.

In order to ascertain the maximal possible fluctuation in weight attributable to the contents of the alimentary canal and bladder, we permitted six 4 months old mice to fast for 27 hours, depriving them also of water during the last 4 hours of this period. The curve of weight of mice deprived of food shows a sharp break at 6 hours, indicating that at the end of this period the intestines are completely emptied. Hence the alimentary canals of these mice were certainly empty of food at the end of the period indicated and no small proportion of their loss of weight was due to actual inanition. We now weighed the animals, then supplied them with an abundant meal of every variety of food, and replaced their drinking tubes. At the end of 1 hour, when they had ceased eating with voracity, the average gain in weight was 5 per cent of the initial (non-fasting) weight. This, therefore, may be regarded as the extreme possible fluctuation in weight due to varying intestinal and bladder contents. The fluctuations actually encountered must, of course, have been considerably less than this, probably not exceeding 1 or 2 per cent of the body weight.

The animals, as stated, were weighed to the nearest 0.5 gm. subsequently to 4 weeks of age (12 gm. weight in males, 10.5 in females), being weighed to the nearest 0.1 gm. previously to this. From this source, therefore, an error of 0.25 gm. may possibly arise in the recorded weight of an individual mouse. This would lead to an error of 2 per cent in the recorded weight of a mouse of 4 or 5 weeks of age, and less than 1 per cent in the recorded weight of an adult mouse. The error thus introduced into the mean weight of a number of mice would, of course, be much less than this, since in the long run plus and minus errors due to this source of inaccuracy would tend to cancel each other.

SUMMARY.

Methods employed in experiments on the growth of mice hereafter detailed are described by which the animals may be maintained in good health and as free as possible from zymotic disease and other deleterious factors in the environment which might conceivably exert an effect upon their growth. Improved forms of cages, food receptacles, and nest houses are described.

Rapid methods of handling and weighing large numbers of animals, and a balance especially adapted to this purpose, are also described.

EXPERIMENTAL STUDIES ON GROWTH.

II. THE NORMAL GROWTH OF THE WHITE MOUSE.

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INTRODUCTION.

Nature of the Growth Process.

I have shown in previous communications[1] that the growth of man and of animals consists of a number of phases or growth cycles which succeed one another and to some extent merge into one another at the transitional period. These transitional periods are critical periods in the growth of the animal, and if for any reason accurate linkage with the succeeding cycle fails, the life of the animal or individual is imperilled.

Each of these cycles of growth is characterized by an initial period of slow growth succeeded by a period of rapid growth, and that in turn by a period of slow growth, the entire cycle forming a single S-shaped curve which is symmetrical about its center or moment of maximum velocity.

The chemical syntheses which constitute the growth of an animal are therefore of such a nature that during the first half of any given growth cycle the velocity of synthesis is progressively increased in proportion as it has already proceeded; in other words, it is autocatalyzed or self-accelerated and, like all autocatalyzed reactions, in the latter half of the cycle the syntheses are retarded by the progressive accumulation and mass action of the products of the synthesis. In the communications to which

[1] Robertson, T. B., *Arch. Entwcklngsmechn. Organ.*, 1908, xxv, 581; 1908, xxvi, 108; 1913, xxxvii, 497; *Univ. California Publications, Physiology*, 1914–15, iv, 211; *Am. J. Physiol.*, 1915, xxxvii, 1, 74.

reference has been made, it has been shown that the formula of autocatalysis,

$$\text{Log}\frac{x}{A-x} = K\,(t - t_1)$$

(where x is the increment in weight attained in any one cycle at time t, A is the total increment in weight attained in the cycle, t_1 is the time at which the cycle is half completed, and K is a constant expressing the specific velocity of the process), accurately represents the quantitative relationships which have been observed in those instances where accurate measurements made upon a sufficient number of individuals have been available.

Catalysers of Growth. Objects of the Experiments.

The fact that the syntheses which constitute the growth of an organism are autocatalyzed, implies the existence of substances which are capable of acting as catalysers of growth. Nor is direct evidence lacking to this effect. We know that many of the glands of internal secretion, notably the anterior lobe of the pituitary body, the thyroid, and the thymus, are capable of exerting important influences upon the processes of growth,—influences the lack of which becomes apparent in ways which are now familiar when one of these glands is removed by operation or injured by disease. These glands are universally supposed to exert their influence by means of secretions which are thrown into the blood stream and which must therefore contain positive or negative catalysers of certain phases or types of growth. Moreover, direct evidence has been adduced by various investigators to show that certain substances, notably lecithin and cholesterol, exert an accelerating or retarding influence upon certain types of growth.[2]

In this connection it is a fact of great significance that both lecithin and cholesterol, while notably accelerating certain types

[2] King, H. D., *Biol. Bull.*, 1907, xiii, 40. Johnson, M., *Univ. California Publications, Zoology*, 1913, xi, No. 4. Bain, W., *Lancet*, 1913, clxxxii, 918. Robertson, T. B., *Arch. Entwcklngsmechn. Organ.*, 1913, xxxvii, 497. Robertson, T. B., and Burnett, T. C., *Proc. Soc. Exp. Biol. and Med.*, 1912-13, x, 140; *J. Exp. Med.*, 1913, xvii, 344. Browder, A., *Univ. California Publications, Physiology*, 1915, v, 1.

of growth, exert a no less notable retardation upon certain other types, and, furthermore, that those types of growth which are accelerated by cholesterol are retarded by lecithin, and *vice versa*.

This fact leads directly to the conception outlined in a previous communication;[3] namely, that of the occurrence of two different types of growth which I have designated the autokinetic and the autostatic types, respectively, the former type being that in which the growth accelerator progressively increases in amount, the latter that in which it progressively diminishes in amount; either process is, as I have shown in the communication to which reference has been made, quantitatively expressible by the autocatalytic formula. For reasons which are described in detail in the same article, increase in the concentration or available mass of the catalyser may be expected to lead to an acceleration of the latter half of an autostatic or the initial half of an autokinetic cycle, a period of growth which we may inclusively term the "anatachytous phase," while a like increase in the concentration or available mass of the catalyser may be expected to lead to the retardation of the latter half of an autokinetic or the initial half of an autostatic cycle, a period which we may in like manner inclusively term the "katatachytous phase" of growth (ταχυτής, velocity).

The possibility, or rather probability must not, of course, be lost sight of that many different catalysers of growth exist, each of which may not improbably be furnished to the organism from a different source (*e.g.*, gland of internal secretion). Nor is it at all unlikely that the growth of certain tissues, such as the subcutaneous or the bony tissues, may be controlled by special catalysers of their own. Furthermore, specific retarders of certain phases of growth may exist, acting either directly as negative catalysers, or indirectly by diminishing the available mass of or rendering inoperative a positive catalyser of growth. Nevertheless, broadly speaking, the action of all these substances may be expected to conform to the general characteristics outlined above.

The object of the present series of experiments is to elucidate if possible, by the direct administration of substances known or suspected to exert an influence upon growth, the chemical nature

[3] Robertson, *Arch. Entwcklngsmechn. Organ.*, 1913, xxxvii, 497.

and mode of action of the various catalysers of growth. Owing to its hardiness, rapid growth, smallness, and fecundity, the white mouse was chosen as the experimental animal. But for this purpose it was necessary to establish an accurate norm or curve of growth of normal animals obtained under conditions precisely resembling those which prevailed among the animals receiving the known or suspected catalysers. Such a norm for the white mouse is not available, and in any case, if previously established, might not necessarily apply to the race and environment employed in this experiment. The present article is designed to present the data which furnish such a norm. In addition to the growth, other factors in the life history of the animals were studied, with a view to making the accumulated data as complete a résumé of the changes involved in the growth of these animals as it was feasible to obtain.

Methods of Breeding, Weighing, etc.

The breeding animals were kept in oil cans perforated at the bottom, with one side cut out and covered with wire gauze. Wooden nest boxes were employed. In all other respects, save that they were fed with finely chopped hard boiled egg instead of with raw egg, the animals were treated in exactly the same way as the experimental animals.[4] Pregnant animals were placed in cans by themselves as soon as their condition was discovered.

The young were weaned at 3 weeks after birth, as recommended by Daniel,[5] and from 1 to 2 weeks later, *i.e.*, at the age of 4 to 5 weeks, they were removed to the cages described in the preceding article and handled and fed as therein stated, the sexes being segregated.

The animals were weighed upon the day of birth and at 7 days, without any attempt at differentiation of the sexes. Thereafter, the animals were marked with spots of methylene blue.[6] The records of each animal were kept separate and the sex of

[4] Compare the preceding article on Methods.

[5] Daniel, J. F., *Am. Naturalist*, 1912, xlvi, 591.

[6] At this age, when the hair begins to grow, it is easy to mark the animals in this way if the hair be first moistened with a little 95 per cent alcohol and then stained with a concentrated aqueous solution of methylene blue. These marks last several weeks.

each animal was determined when the sexual organs became sufficiently differentiated to render this an easy matter. Weighings were repeated on the 11th (9th to 13th inclusive), 14th (exact), 17th (15th to 19th inclusive), 23rd (22nd to 24th inclusive), and 28th (25th to 31st inclusive) days, and thereafter once a week until the age of 210 days, after which the weighings were biweekly. Animals weighed on the 25th to the 31st days, inclusive, after birth, were considered to have been weighed on the 28th day, those weighed on the 32nd to the 38th days inclusive to have been weighed on the 35th day, and so forth. Animals weighed on the 218th to the 231st days inclusive were considered to have been weighed on the 224th day, animals weighed on the 232nd to the 245th days inclusive to have been weighed on the 238th day, and so forth, the animals weighed being sufficiently numerous and sufficiently scattered over these gaps of time to permit this to be done without introducing any appreciable error. The animals (including and subsequent to the 4th week) were always weighed at the same time of day; namely, the middle of the afternoon.

Period of Gestation. Size of the Litter.

According to Daniel,[5] the period of gestation in the white mouse is 20 days.

From June 19, 1914, to July 25, 1915, inclusive, the total number of animals born in the laboratory was 1,242. The number of litters was 241. Thus the average number of animals born in one litter was 5.15 or, in round numbers, 5.

The standard deviation of the size of the litter was determined in the manner usual in statistical investigations;[7] namely, by squaring the deviation of each litter from the mean (5), adding these squared deviations, dividing the sum by the total number of litters, and taking the square root of the quotient. The standard deviation was in this way found to be 2.2. The percentage ratio of the standard deviation to the mean is the measure of the variability of the quantity measured. It expresses the percentage of deviation upon either side of the mean within which two-thirds of the observed measurements may be expected to

[7] Davenport, C. B., Statistical Methods, New York, 2nd edition, 1904, 15. Yule, G. U., An Introduction to the Theory of Statistics, London, 2nd edition, 1912, Chapter 8.

lie.[8] In the case of the size of the litter delivered by the white mouse the variability is extremely high, no less than 42.8 per cent, which means that two-thirds of any large number of litters may be expected to lie between 2.95 and 7.35. In round numbers over two-thirds of the litters may be expected to consist of 3, 4, 5, 6, or 7 young. The largest observed litter contained 11 young.

Of all the animals born in the laboratory during the period mentioned, 358, or 29 per cent, died from various causes (inanition, paratyphoid, being eaten or otherwise injured by the mother) before they were weaned.

There was a noticeable tendency for some large litters to suffer from lack of nutrition, especially towards the end of the lactation period. Occasionally such litters were divided up among other nursing mothers, with good results. Animals that were noticeably abnormal or in poor condition were, of course, not utilized for weight measurements. In order to avoid any possible selection, the litters were taken by chance, one class of experimental animals (*i.e.*, normals, pituitary-fed, etc.) being usually completely filled (with the exception of those subsequently added to replace early deaths from paratyphoid) before another class was started. Hence the norm herein presented offers as nearly as possible a true picture of the normal growth of all the experimental animals.

Growth Curve of Normal White Mice.

Tables I and II show the average weights at the ages chosen of male and female mice respectively, the figures for the weights at birth and 7 days referring to mixed males and females. The variability of the weight at each age, determined in the manner described above, is included in the third and the number of animals weighed in the fourth columns of each table. These results are also depicted graphically in Figs. 1 and 2, the abscissæ being time and ordinates weight, the smooth curves representing the progressive alterations in weight and the irregular curves the alterations in variability (ordinates, percentage) with age. In the growth curves the dots represent the actually determined

[8] More exactly, 68.27 per cent; compare Davenport, Statistical Methods, New York, 2nd edition, 1904, 16.

weights; in the variability curves the observed points are joined by straight lines.

The following characteristics may be especially noted in these curves: In each sex there are three separate extra-uterine growth

TABLE I.

Normal Male Mice.

Age.	Weight.	Variability.	No. weighed.	Age.	Weight.	Variability.	No. weighed.
	gm.	*per cent*		*wks.*	*gm.*	*per cent*	
At birth.	1.23	11.4	56	21	26.28	11.2	35
days				22	26.06	9.0	35
7	3.31	19.2	45	23	26.34	9.8	34
11	4.67	19.3	41	24	26.82	10.1	34
14	5.14	19.1	24	25	27.05	11.0	33
17	5.70	24.7	43	26	26.94	10.8	33
23	9.32	18.5	45	27	26.55	11.3	32
wks.				28	27.19	10.9	31
4	12.38	24.4	65	29	27.08	10.9	31
5	12.45	24.6	117	30	27.23	9.5	31
6	15.58	22.0	72	32	27.61	9.6	32
7	18.08	16.9	43	34	27.71	10.5	31
8	19.36	15.9	42	36	27.95	10.1	30
9	20.63	16.5	42	38	28.20	10.5	30
10	21.19	16.7	42	40	28.27	10.4	30
11	21.81	13.3	42	42	28.28	11.2	30
12	22.65	13.7	42	44	28.62	12.0	30
13	23.31	14.2	37	46	28.55	11.7	30
14	23.96	14.1	36	48	28.22	10.8	30
15	24.28	11.9	36	50	28.88	11.1	30
16	24.75	12.7	36	52	28.68	10.7	30
17	25.21	12.4	36	54	28.67	12.0	30
18	25.61	12.1	36	56	29.32	12.0	30
19	25.81	12.1	35	58	29.10	10.9	30
20	26.10	10.8	35	60	29.08	11.8	30

cycles, just as in the growth curve of human beings.[9] In the males the first cycle (I) attains its maximum velocity at some time between birth and 7 days, nearer the latter than the former, and culminates at 14 days. The second cycle (II) begins coincidently with the culmination of the first, attains its maximum velocity at 21 to 23 days, and culminates soon after the 28th day.

[9] Robertson, *Am. J. Physiol.*, 1915, xxxvii, 1.

Here a definite pause in growth occurs, but the third growth cycle (III) begins to show its effects within the succeeding week, attains its maximum velocity rather rapidly, at about 6 weeks, and thereafter decreases continuously but slowly in velocity so that

TABLE II.

Normal Female Mice.

Age.	Weight.	Variability.	No. weighed.	Age.	Weight.	Variability.	No. weighed.
	gm.	*per cent*		*wks.*	*gm.*	*per cent*	
At birth.	1.23	11.4	56	21	23.06	12.3	36
days				22	23.32	12.5	36
7	3.31	19.2	45	23	23.51	12.0	35
11	4.58	23.0	33	24	23.68	11.3	36
14	4.91	22.5	17	25	23.79	11.4	35
17	5.48	25.3	35	26	24.04	11.6	36
23	8.45	18.2	37	27	24.00	11.8	35
wks.				28	23.58	10.3	36
4	10.39	23.4	89	29	23.84	12.2	35
5	11.81	19.4	77	30	23.92	12.4	36
6	14.12	18.9	68	32	24.18	11.5	36
7	16.77	15.0	37	34	24.18	12.2	36
8	17.99	13.9	36	36	24.65	11.2	36
9	18.78	13.4	36	38	24.80	12.3	36
10	19.38	14.3	36	40	25.03	11.9	35
11	20.04	12.6	36	42	25.07	13.1	35
12	20.31	12.6	36	44	25.52	15.3	34
13	21.04	13.0	36	46	25.68	14.6	33
14	21.21	13.2	36	48	25.45	14.6	32
15	21.78	13.7	36	50	25.50	14.0	32
16	22.14	12.6	36	52	25.76	15.3	29
17	22.29	11.9	36	54	25.78	15.8	29
18	22.22	11.9	36	56	26.00	16.5	29
19	22.60	12.0	36	58	26.26	16.7	29
20	22.60	11.2	36	60	26.12	17.8	29

growth of the animals is still occurring, though with extreme slowness, even between the 50th and 60th weeks succeeding birth.

In females the succession of events is very similar, save that the second cycle merges more gradually into the third, which begins to exert a noticeable effect upon the growth of the animal at a somewhat earlier age than in the male.

All the cycles are less extensive in the female than the male,

the growth curve of the female lying completely under that of the male. The difference in favor of the male at the end of Cycle

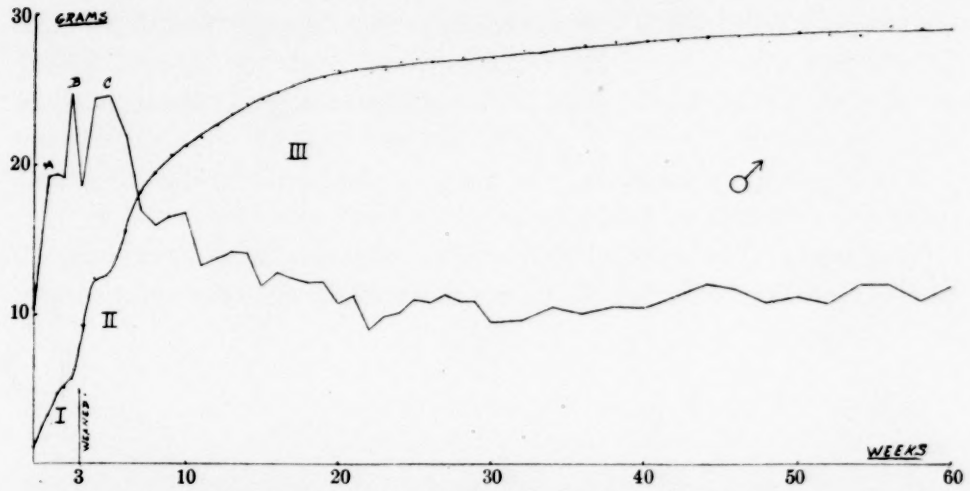

FIG. 1. Changes in weight (smooth curve) and variability (irregular curve) of normal male mice with age. Ordinates of the growth curve, gm.; the dots represent actual observations. Ordinates of the variability curve represent percentages.

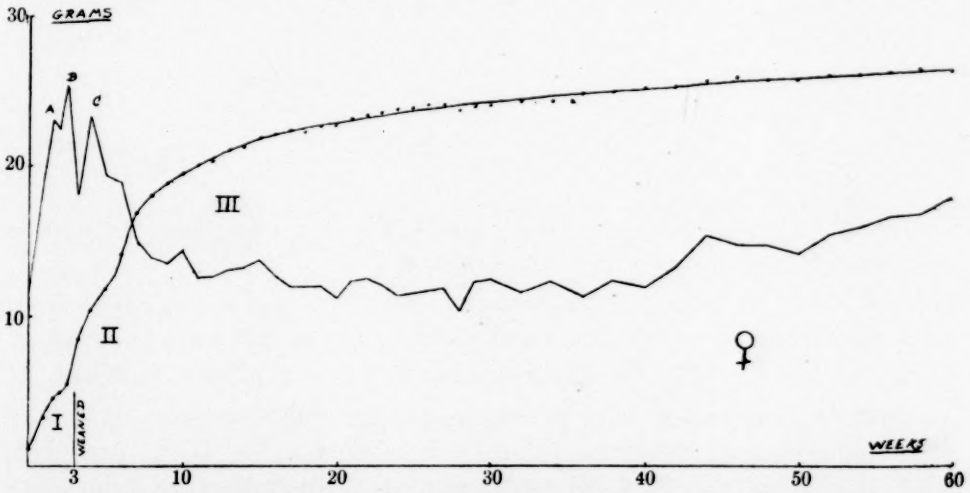

FIG. 2. Changes in weight (smooth curve) and variability (irregular curve) of normal female mice with age. Ordinates of the growth curve, gm.; the dots represent actual observations. Ordinates of the variability curve represent percentages.

I (14 days) is 0.23 gm.; at the end of Cycle II (5 weeks) it is 0.64 gm.; and at 60 weeks it is 2.96 gm.

All the observed points lie almost exactly upon the smooth curves. But a slight tendency to depart therefrom is distinctly noticeable in both sexes at about the 25th week; very slight oscillations in the growth curve make their appearance here and afterwards disappear. It would appear that the age of about 25 weeks may be a period of relative instability in the growth of these animals.

The algebraic formulation of these curves will be deferred to a subsequent article of this series.

The variability curves display an unmistakable tendency of the variability to increase with increasing velocity of growth,

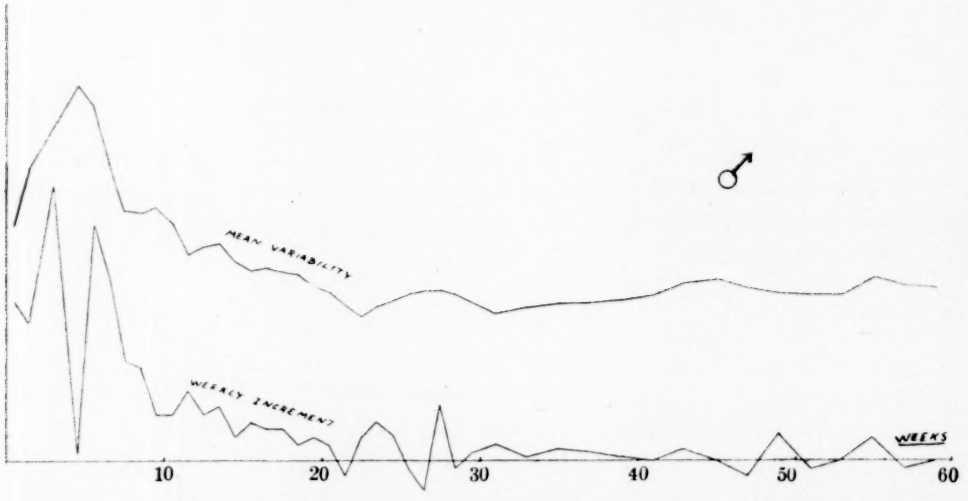

FIG. 3. Comparison of the mean variability (per cent) and the weekly increment in weight (gm.) of normal male mice.

and to decrease with decreasing velocity of growth. Thus in each sex the variability curve displays three maxima, marked A, B, and C, corresponding more or less closely in time with the three maxima of growth velocity due to Cycles I, II, and III, and thereafter the variability curve gradually falls to a relatively steady minimum value as the velocity of growth gradually decreases.

This relationship of variability to velocity of growth is clearly displayed in Figs. 3 and 4 in which the curve of mean variability is compared with the curve of weekly increments for males and females respectively. In constructing these curves the increment

from the end of the 5th to the end of the 6th weeks, for example, is regarded as the rate of weekly increment at the middle of the 6th week, while the mean of the variabilities at the end of the 5th and 6th weeks is regarded as the mean variability at the middle of the 6th week. It will be seen that the two curves run closely parallel to one another; in other words, the changes in variability are approximately proportional to the changes in weekly increment.

It will be noticed, however, that there is a distinct tendency for the variability of both sexes, but especially of the females, to undergo a progressive increase after the 40th week, without

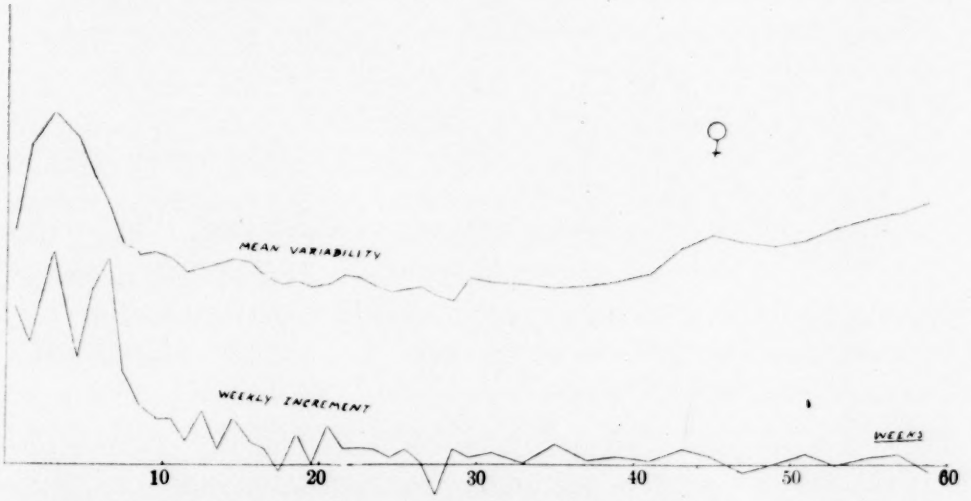

FIG. 4. Comparison of the mean variability (per cent) and the weekly increment in weight (gm.) of normal female mice.

any corresponding increase in the magnitude of the weekly increment.

Confirmation of Daniel's Estimate of the Period of Lactation.

It will be observed, on examining the growth-curves in Figs. 1 and 2, in which the end of the period of lactation is indicated by a vertical line, that the pause in growth which accompanies the culmination of Cycle I precedes by a distinct interval (1 week) the time at which the animals are weaned. Therefore this pause is not due to insufficient nourishment following the cessation of the supply of mother's milk. Furthermore the acceleration due to

the initial stages of the second cycle is already well under way before weaning occurs, and is therefore not attributable to the greater variety of diet enforced on the animals by the deprivation of milk. In fact the weaning of the animals is accompanied by not the slightest distortion of the growth curve which passes smoothly and with a steep slope through the point corresponding to the date of weaning. It is clear, therefore, that weaning on the 21st day after birth results in no physiological disturbance whatever in the young.

Estimate of the Number of Animals Required in Growth Experiments.

From the estimates of variability in weight at different ages which are tabulated above, it is possible to estimate the number of animals of a given sex and age which must necessarily be employed to obtain a measure of average weight which shall possess any desired degree of accuracy.

The *probable error* ϵ in the estimate of the mean, that is, the error which is as likely as not to occur, is expressed by:

$$\frac{0.6745\,\sigma}{\sqrt{N}} = \epsilon$$

where σ is the standard deviation estimated in the manner described above, and N is the number of measurements made; *i.e.*, animals weighed.[7] The variabilities tabulated above are $\frac{\sigma}{W} \times 100$ where W is the mean weight for the given age and sex.

Now the variability in weight of normal male mice at 40 weeks of age is about 10 per cent, and the mean weight is 28.3 gm. Hence,

$$\sigma = 2.83 \text{ and } \epsilon = \frac{0.6745 \times 2.83}{\sqrt{N}}.$$

If it is desired to make ϵ of any required dimensions, for instance 1 per cent, we can insert this value of ϵ in the formula and calculate therefrom N or the number of animals which we must employ in order to obtain an estimate of the mean weight which will probably lie within 1 per cent of the true value.

In the present instance, if ϵ is 1 per cent of the mean weight of the animals at 40 weeks we have:

$$\frac{0.6745 \times 2.83}{\sqrt{N}} = 0.283$$

whence $N = 45$. In other words, we see that in order to obtain an estimate of the mean weight of males at 40 weeks of age which shall as likely as not lie within 1 per cent of the true value, we must weigh no fewer than 45 animals.

In order to obtain an estimate of the mean weight which shall just as likely as not lie within 2 per cent of the true value, we must weigh 14 animals.

The variability of normal female mice at 40 weeks of age is about 12 per cent, and their mean weight 25 gm., from which we find that in order to obtain an estimate of the mean weight of females at 40 weeks of age which shall just as likely as not lie within 1 per cent of the true value we must employ 65 animals. If we are content with an estimate which may differ by 2 per cent from the true value we may employ 16 animals.

At 5 weeks the variability of males is about 25 per cent and the mean weight 12.45 gm. Hence the number of animals required to obtain an estimate of weight subject to a probable error of 1 per cent at this age is no less than 282. If we are content with a probable error of 2 per cent we may employ 71 animals.

For females at 5 weeks (variability, 20 per cent; mean weight, 10.39 gm.) the corresponding numbers are 183 and 46.

In the measurements tabulated above, and in general throughout the growth experiments to be described in this series, the number of animals of one sex and experimental class employed was, with few exceptions, intermediate between the number necessary to secure a probable accuracy of 1 per cent and that necessary to secure a probable accuracy of 2 per cent.

In the preceding article on methods it is shown that the probable error in individual weighings due to the fluctuating contents of the alimentary canal, and also that due to the weighings (subsequent to 4 weeks of age) being expressed to the nearest 0.5 gm., are approximately of the same magnitude; namely, from 1 to 2 per cent.

From these estimates it may be clearly seen how little reliance may be placed upon experimental conclusions concerning growth, published in not a few quarters, which are based on measurements made upon 2 to 6 animals.

External Changes in Mice Which Accompany Their Growth.

According to Daniel,[5] "Between birth and maturity four well defined stages occur. . . . The *first stage* is that in which the newly born young have a peculiarly red and transparent skin through which is seen the stomach white with milk. Following this at the end of the sixth or seventh day a *second stage* is evident in which the body is covered with flaky scales of dandruff,—forerunners of a coat of silky fur. A *third important stage*. . . . is usually shown on the ninth or tenth day at which time the mammæ in the young females appear. These can be observed for an interval up to the thirteenth or fourteenth day, at which time the fur usually obscures them. . . . The *fourth period*, on the fourteenth day, is characterized by the advent of sight. . . . The regularity with which this period occurs. . . . is a sufficiently exact criterion to make it an index of age."

I can add my testimony to the above as to the extraordinary invariability of the advent of sight on the 14th day after birth. Unusually small and unusually large animals open their eyes upon the same day. In a very few cases I have observed the eyes to open on the 13th day and in equally few cases not until the 15th, but in the great majority of cases the eyes open precisely on the 14th day after birth. It will be noted that this phenomenon, together with the attainment of a full coat of fur, coincides exactly with the termination of the first cycle of growth.

Birth occurs during the first half of the first growth cycle, exactly as it does in human beings.[9]

The vagina opens at about the 6th week and individuals may occasionally pair at this age, although usually not until later. The attainment of puberty, therefore, coincides with the period of maximum velocity of growth in the third growth cycle, exactly as it does in human beings.[10]

[10] Robertson, *Arch. Entwcklngsmechn. Organ.*, 1908, xxv, 581; *Am. J. Physiol.*, 1915, xxxvii, 1.

Changes in the Thyroid and Thymus Which Accompany Growth.

The anterior lobe of the pituitary body, the pineal body, the thyroid, and the thymus are known to exert notable influences upon the growth of animals. Their development in relation to the development of the animal as a whole is therefore of great interest. The pituitary and pineal bodies in such small animals as mice are inaccessible to measurement, but the thyroid and the thymus may readily be dissected out and weighed. This was accordingly done at certain definitely selected ages: at birth (10 individuals without distinction of sex); at 14 days, *i.e.*, at the culmination of the first growth cycle (10 males, 11 females); at 35 days, *i.e.*, at the culmination of the second growth cycle (10 males, 10 females); at 70 days (10 males, 10 females); and at 210 days (6 males, 6 females).

TABLE III.

Absolute Weights of Thyroid and Thymus.

Age.	Thyroid. ♂	Thymus. ♂	Thyroid. ♀	Thymus. ♀
	gm.	*gm.*	*gm.*	*gm.*
At birth	0.006	0.004	0.006	0.004
14 days	0.032	0.030	0.031	0.029
35 "	0.112	0.061	0.095	0.071
70 "	0.187	0.049	0.137	0.064
210 "	0.208	0.031	0.179	0.045

The animals for this purpose were selected at random from our general stock and had been fed and treated in exactly the same way as other normal animals. At the required ages they were weighed and then chloroformed. They were then dissected and the thyroid and thymus immediately weighed to the nearest mg.

The thyroid in the mouse is a large triangular shaped organ, the apex of the triangle being directed ventrally and posteriorly, and the two extremities of the base being surmounted by small hemispherical elevations differing somewhat in appearance from the rest of the gland. A longitudinal furrow indistinctly divides the gland into right and left lobes.

The thymus is a distinctly lobated organ situated in the thoracic cavity just ventral and somewhat anterior to the heart.

Table III summarizes the observed absolute weights of these

glands in males and females at the ages enumerated, the figures for the weights at birth referring to males and females without distinction of sex.

It will be observed that during the first cycle of growth the thyroid and thymus both increase considerably in weight and to about the same extent. During the second cycle the thyroid increases much faster than the thymus. After the culmination of the second cycle of growth the thymus decreases markedly in weight in both sexes, while the thyroid continues to grow. Up to the culmination of the first cycle of growth the thyroid and thymus are practically of equal weight in both sexes, but subsequently to this the thyroid grows more rapidly in the male than

TABLE IV.

Weights of Thyroid and Thymus in Relation to Body Weight.

Age.	Thyroid. ♂	Thymus. ♂	Thyroid. ♀	Thymus. ♀
	per cent	*per cent*	*per cent*	*per cent*
At birth	0.47	0.30	0.47	0.30
14 days	0.71	0.68	0.71	0.65
35 "	0.78	0.45	0.79	0.60
70 "	0.90	0.24	0.73	0.34
210 "	0.77	0.12	0.80	0.20

in the female, while the thymus grows more rapidly in the female than in the male.

In Table IV the weights of the thyroid and thymus are expressed in mean percentages of the total body weights of the individuals from which they were taken.

It will be observed that during the first cycle of growth the thyroid and thymus both increase more rapidly than the body weight. Subsequently to this the thyroid increases almost in direct proportion to the body weight, while the thymus increases less rapidly than the body weight, or decreases.

The development of these glands therefore presents well marked stages which coincide closely with the cycles displayed in the growth curve of the animals.

Changes in the Alcohol-Soluble Phospholipoid Content of the Tissues during Growth.

Several investigations to which reference has already been made having shown that the phospholipoids and also certain other alcohol-soluble phosphorus-containing substances[11] exert a decided influence upon certain types of growth, an attempt was made to ascertain the changes in the content of this type of substances in the tissues of the mouse which accompany growth. For this purpose the animals used in estimating the weights of the thyroid and thymus were employed, the glands being restored to the carcases after weighing. The animals were decapitated, it being thought that the extensive phospholipoid content of the cerebral tissues, where lipoids perform the unusual function of constituting structural elements, would confuse the relationship between the lipoid content and the stage of development which might conceivably subsist in other tissues. The decapitated carcases were minced twice over in a small mincing machine, the minced tissue was weighed and then transferred quantitatively to a mortar in which it was thoroughly ground up with three times its weight of a mixture of equal parts by weight of anhydrous sodium and calcium sulfates. This mixture was then quantitatively transferred to a shallow dish and dried on a water bath. It was stirred from time to time to prevent caking. Drying under these conditions[12] is very rapid, being complete in about 1 hour. The dried mass was then transferred again to the mortar and thoroughly pulverized.

This pulverized material was quantitatively transferred to alundum extraction thimbles 30 mm. in diameter and 54 mm. high, which were placed in the flasks of a Bailey-Walker extraction apparatus,[13] the flasks being 1 inch longer than in the model usually supplied.[14] The alundum thimbles were made by cutting down the ordinary 80 by 30 mm. type to 54 mm. in length, drilling three small holes near to the top, and twisting into these small lengths of aluminum wire, which projected outwards and by contact with the neck of the flask held the thimbles upright,

[11] Compare article No. IV in this series.

[12] Compare Leathes, J. B., The Fats, London, 1910, 53.

[13] Walker, P. H., and Bailey, L. H., *J. Ind. and Eng. Chem.*, 1914, vi, 497.

[14] By Eimer and Amend, New York.

the bottom of the thimbles resting on the indentations at the bottom of the neck of the flask and just over the boiling solvent employed in the extraction. This apparatus has the double advantage of carrying out the extraction at the boiling temperature of the solvent, and of very rapid and simple manipulation.

The material was extracted continuously with boiling alcohol for 48 hours. The combined alcoholic extracts from the material derived from 10 mice (11 in the case of 14 day females, 6 each in the cases of 210 day males and females) were measured and an

TABLE V.

Alcohol-Soluble Phosphorus in Gm. per 100,000 Gm. of Fresh Tissue.

Age.	♂	♀
At birth. *days*	91, 96 Mean = 94	91, 96 Mean = 94
14	92	90, 94 Mean = 92
35	88, 85 Mean = 87	91, 88 Mean = 90
70	77	79, 80 Mean = 80
210	62, 57 Mean = 60	65, 66 Mean = 66

aliquot portion of each combined extract corresponding to 5 gm. of the freshly minced tissue (2.5 gm. in the case of the mice at birth) was evaporated by placing it in a Kjeldahl digestion flask and plunging the flask into boiling water to a depth exceeding that of the contained liquid.

The residue was ashed with Neumann's sulfuric-nitric acid mixture and the phosphorus determined by von Wendt's modification of Neumann's method.[15] In each case, excepting the 14 and 70 day males, the estimations were made in duplicate. Table V shows the results obtained.

[15] Von Wendt, G., *Skandin. Arch. Physiol.*, 1905, xvii, 217.

It will be observed that there is a steady decrease in the alcohol-soluble phosphorus in the tissues of mice from birth onwards, and almost exactly in direct proportion to their age. It should be noted that were this rate of decrease to be maintained the alcohol-soluble phosphorus content of the tissues would fall to zero at 630 days; *i.e.*, at 21 months after birth.

These results differ strikingly from those reported by Siwertzow[16] in human beings. This observer states that the alcohol-soluble phosphorus in certain specified organs undergoes marked fluctuations with age, increasing in infants up to the age of 2 years and thereafter decreasing markedly. It is possible that these results merely indicate a transfer of alcohol-soluble phosphorus from one organ to another, the tissues of the animal as a whole becoming continuously poorer in this substance. It is also possible that the P : N ratio in the alcohol extract might reveal fluctuations bearing some relation to the growth cycles. This question is being investigated and will form the subject of a subsequent communication.

Analysis of the Causes of Death.

Between the 10th and 60th weeks six deaths were recorded among 36 normal males[17] and 7 among 36 normal females. The following is a summary of the causes of death:

Males.

Injuries received in fighting	2
Accidental injuries	2
Unknown causes	2
Total	6

Females.

Injuries	1
Pneumonia	3
Cancer	2
Unknown causes	1
Total	7

[16] Siwertzow, D. I., Dissertation, St. Petersburg, 1904; cited after *Biochem. Z.*, 1904, ii, 310.

[17] This excludes the six males in one compartment which simultaneously developed paratyphoid at the unusually advanced age of 17 weeks and were replaced. The weight data referring to these animals were utilized in Table I up to the 12th week and omitted thereafter.

SUMMARY.

1. The curves of growth and variability of the white mouse have been determined from birth to the end of the 60th week succeeding birth under conditions and with stock exactly resembling those employed in the experiments to be detailed in subsequent communications of this series.

2. The average number of animals born in one litter is 5.15. The variability of the number born in a litter is very high; namely, 42.8 per cent. 29 per cent of the animals born in the laboratory died from various causes before being weaned on the 21st day after birth.

3. In each sex there are three separate extra-uterine growth cycles. The first cycle attains its maximum velocity at some time shortly prior to 7 days after birth and culminates at 14 days. The second cycle attains its maximum velocity at 21 to 23 days and culminates soon after the 28th day. The third cycle attains its maximum velocity at about 6 weeks and thereafter decreases in velocity continuously but very slowly, so that growth of the animals still occurs between the 50th and 60th weeks succeeding birth.

4. All the cycles are less extensive in the female than in the male.

5. The variability in weight of the animals shows a decided tendency to increase with increasing velocity of growth and to decrease with decreasing velocity of growth. After the 40th week, however, especially in the females, there is a tendency of the variability to increase progressively without any corresponding increase in the rate of growth.

6. Weaning of the animals on the 21st day after birth produces no physiological disturbance whatever in the young.

7. It is shown from the variability of the weight that considerable numbers of animals must be employed to obtain reliable data in experiments upon growth. The weight data reported in this and succeeding articles are probably within 1 or 2 per cent of the true values.

8. Birth in mice occurs during the first half of the first growth cycle. The eyes open coincidently with the culmination of the first growth cycle, a complete coat of fur being acquired at the same time. Puberty coincides with the period of maximum velocity of growth due to the third growth cycle.

9. The development of the thyroid and of the thymus in mice presents well marked stages which coincide closely with the cycles displayed in the growth curve of these animals. Subsequent to the culmination of the first growth cycle the thyroid is heavier in the male than in the female, while the thymus is heavier in the female than in the male.

10. There is a steady decrease in the percentage content of alcohol-soluble phosphorus in the tissues (other than cerebral tissues) of mice from birth to 210 days of age, and this decrease is almost exactly in direct proportion to their age.

EXPERIMENTAL STUDIES ON GROWTH.

III. THE INFLUENCE OF THE ANTERIOR LOBE OF THE PITUITARY BODY UPON THE GROWTH OF THE WHITE MOUSE.

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(Received for publication, January 28, 1916.)

Present Knowledge. Objects of the Experiments.

The almost unanimous experience of observers is to the effect that the administration of the pituitary body either by mouth or hypodermically to young animals causes retardation of their growth.[1] This retardation is due to the anterior lobe, since anterior lobe alone produces retardation,[2] while the posterior lobe alone does not produce this effect upon growth.[3]

The results obtained by Schäfer[4] with rats are of significance when viewed in the light of the experimental results about to be enumerated. Very small numbers of animals were employed (three or four in each group), but the general result was that the early effect of feeding young animals with pituitary tissue was to cause retardation of growth, while later, that is when administration had been delayed until the animals were half grown, no such retardation and even some acceleration was displayed. Looking to the gross effect of these administrations (only continued

[1] Caselli, A., *Riv. sper. freniat.*, Reggio-Emilia, 1900, xxvi, 176, 486. Fodera, F. A., and Pittau, L., *Gazz. med. e chir.*, 1909, viii, 149. Cerletti, U., *Arch. ital. biol.*, 1907, xlvii, 123. Etienne, G., and Parisot, J., *Arch. méd. exp. et d'anat. path.*, 1908, xx, 423. Sandri, O., *Arch. ital. biol.*, 1909, li, 337. Crowe, S. J., Cushing, H., and Homans, J., *Bull. Johns Hopkins Hosp.*, 1910, xxi, 127, cited after Cushing, H., The Pituitary Body and Its Disorders, Philadelphia, 1912.

[2] Aldrich, T. B., *Am. J. Physiol.*, 1912, xxx, 352; 1912–13, xxxi, 94 (in dogs and rats). Wulzen, R., *Am. J. Physiol.*, 1914, xxxiv, 127 (in fowls).

[3] Aldrich, *Am. J. Physiol.*, 1912–13, xxxi, 94.

[4] Schäfer, E. A., *Quart. J. Exp. Physiol.*, 1912, v, 203.

for 3 months), Schäfer concludes that administration of pituitary tissue exerts little or no effect upon growth. Having regard to the fact, to which attention is drawn in the preceding article of this series, that growth catalysers may be expected, and in many instances have been shown to exert totally opposite effects upon growth at different stages of development or upon differing types of growth, we cannot accept this negative conclusion. Schäfer's results, although lacking the conclusiveness which larger numbers of animals would supply, testify to the truth of the opposite conclusion, and in confirmation of his results and of mine which are about to be enumerated, Dr. Wulzen informs me that a fowl which she continued to feed with anterior lobe for a longer period than that covered by the results enumerated in her article (5 or 6 months) finally displayed acceleration of growth and not only caught up with but surpassed the normals in weight. Moreover, Robertson and Burnett[5] have shown that hypodermic administrations of emulsified anterior lobe of the pituitary body cause marked acceleration of the growth of carcinomata in rats.

It will be clearly seen from the above brief review of the hitherto available experimental data upon the influence of the anterior lobe of the pituitary body upon growth, that the precise nature of this influence may be expected to be considerably affected by the stage of development of the animals to which the gland is administered. This view, indeed, finds ample confirmation in the diversity of the clinical manifestations of pituitary insufficiency, and the profound extent to which, as Cushing has exhaustively shown,[6] these manifestations are modified according to whether the pituitary insufficiency or hyperactivity are pre-adolescent or post-adolescent in origin.

It appeared, therefore, advisable to enhance our knowledge of the mode of action of the anterior lobe of the pituitary body upon growth by obtaining exact information as to the position on the normal curve of growth occupied by the periods of development which are retarded and accelerated respectively by administration of this gland. With this object in view the experiments which are about to be described were undertaken.

[5] Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1915, xxi, 280.

[6] Cushing, H., The Pituitary Body and Its Disorders, Philadelphia, 1912.

Description of the Experiments. Mode of Administering the Tissue. Dose Administered.

Average healthy young mice, not less than 4 or more than 5 weeks of age, were chosen at random and without selection from our stock, transferred to the cages described in the first article of this series, and immediately, and thereafter until the age of 60 weeks, fed upon anterior lobe emulsion. 36 males and 36 females were employed for this purpose. In every respect other than in the administration of the tissue these animals were fed and handled exactly as were the normal animals. Their cages were adjacent to the cages containing the normal animals, the two groups of animals were nearly of the same age, and were always weighed at the same time of day (middle afternoon). Hence no possible factor other than the administration of the pituitary tissue could possibly account for the deviation of these animals from the norm. That the observed deviation was not merely due to the presence of meat in the diet, irrespective of its kind, is conclusively proved in the communication which immediately succeeds this, in which it is shown that a substance may be isolated from the anterior lobe of the pituitary body, which, when administered together with a normal meat-free diet, produces exactly the same type of deviation from the norm as that observed in the experiments at present under consideration.

Through the kindness of the Superintendent, Mr. Ralston B. Brown, to whose cooperation with myself and other members of our staff I desire to express our great indebtedness, we obtained a continuous supply of fresh ox pituitaries from the local slaughter house of the Oakland Meat and Packing Company. These were fed to the mice as a rule less than 48 hours after their extraction from the oxen, being kept upon ice for the greater part of the time meanwhile. They were stripped of their connective tissue capsules, and the anterior and posterior lobes separated, the parts connecting the two lobes being detached from the anterior lobes. The anterior lobes were then mashed and ground to a fine pulp between two ground glass surfaces, and this pulp was put into the beaten and strained yolks and whites of eggs in the proportion of one anterior lobe to 10 cc. of egg. This mixture was shaken, allowed to stand on ice over

night, then shaken again thoroughly, and administered to the mice the following morning. 5 cc. of the mixture were administered to each compartment containing 6 animals, so that one anterior lobe was shared among every 12 animals. Weighings of several hundred anterior lobes showed the average weight of one lobe to be 1.5 gm., so that the average dose of fresh pituitary

TABLE I.

Pituitary-Fed Male Mice.

Age.	Weight.		No. weighed. (Pituitary-fed.)	Age.	Weight.		No. weighed. (Pituitary-fed.)
	Normal.	Pituitary-fed.			Normal.	Pituitary-fed.	
wks.	*gm.*	*gm.*		*wks.*	*gm.*	*gm.*	
4	12.38	12.78	20	25	27.05	25.92	36
5	12.45	13.13	36	26	26.94	26.11	36
6	15.58	15.86	36	27	26.55	26.11	36
7	18.08	17.38	36	28	27.19	26.29	34
8	19.36	18.60	36	29	27.08	26.23	35
9	20.63	19.54	36	30	27.23	26.48	33
10	21.19	20.03	36	32	27.61	26.68	36
11	21.81	20.58	36	34	27.71	27.35	34
12	22.65	21.79	36	36	27.95	27.33	32
13	23.31	22.36	36	38	28.20	27.55	32
14	23.96	23.00	36	40	28.27	28.03	32
15	24.28	23.06	36	42	28.28	28.05	31
16	24.75	23.67	36	44	28.62	28.32	31
17	25.21	24.11	36	46	28.55	28.24	31
18	25.61	24.17	36	48	28.22	28.05	30
19	25.81	24.57	36	50	28.88	28.47	30
20	26.10	24.63	36	52	28.68	28.58	30
21	26.28	24.99	36	54	28.67	28.92	30
22	26.06	25.35	36	56	29.32	28.90	30
23	26.34	25.85	36	58	29.10	29.35	30
24	26.82	26.19	36	60	29.08	29.42	30

tissue administered to each animal was 0.125 gm. The egg mixture was greedily eaten by the animals of all experimental classes, so that the consumption of the tissue administered in this way was practically invariably complete, a few shreds of connective tissue being the utmost that was left undevoured on any occasion. It cannot, of course, be stated that each animal invariably received the same dose, but in the long run that was

probably not far from the truth. The proportionate dose decreased with age, for it was not increased as the animals grew heavier. It was also somewhat larger for the females than for the males, since the females are somewhat less heavy than the males.

RESULTS.

The weights of these and of normal animals of the same ages are compared in Tables I and II and graphically illustrated in

TABLE II.

Pituitary-Fed Female Mice.

Age.	Weight.		No. weighed. (Pituitary-fed.)	Age.	Weight.		No. weighed. (Pituitary-fed.)
	Normal.	Pituitary-fed.			Normal.	Pituitary-fed.	
wks.	*gm.*	*gm.*		*wks.*	*gm.*	*gm.*	
4	10.39	9.53	31	25	23.79	22.04	35
5	11.81	11.76	35	26	24.04	22.10	36
6	14.12	13.89	35	27	24.00	22.29	35
7	16.77	14.71	36	28	23.58	22.54	36
8	17.99	16.24	36	29	23.84	22.39	35
9	18.78	17.22	36	30	23.92	22.91	34
10	19.38	17.93	36	32	24.18	23.07	36
11	20.04	18.24	36	34	24.18	23.42	36
12	20.31	18.88	36	36	24.65	23.63	36
13	21.04	19.21	36	38	24.80	24.01	36
14	21.21	19.57	36	40	25.03	24.38	36
15	21.78	19.72	36	42	25.07	24.44	36
16	22.14	19.90	36	44	25.52	24.62	34
17	22.29	20.49	36	46	25.68	24.68	34
18	22.22	20.72	36	48	25.45	24.74	34
19	22.60	21.16	35	50	25.50	25.38	34
20	22.60	21.21	36	52	25.76	25.71	34
21	23.06	21.34	35	54	25.78	25.82	33
22	23.32	21.67	36	56	26.00	26.06	33
23	23.51	21.67	35	58	26.26	26.26	33
24	23.68	22.07	36	60	26.12	26.65	31

Figs. 1 and 2, the undotted curves representing the normal growth of white mice during the experimental period, the dotted curves the growth of the animals which received the pituitary tissue. The dots represent the positions of the actual observations.

The effect of the administration is qualitatively the same in both sexes, but quantitatively greater in the females. Between the 6th and the 20th weeks growth is markedly retarded, so much so that at 20 weeks of age the pituitary males lag 1.47 gm., and the females 1.39 gm. behind the normals. Between the 20th and the 60th weeks, however, the growth of the pituitary-fed animals is markedly accelerated, so that they not only catch

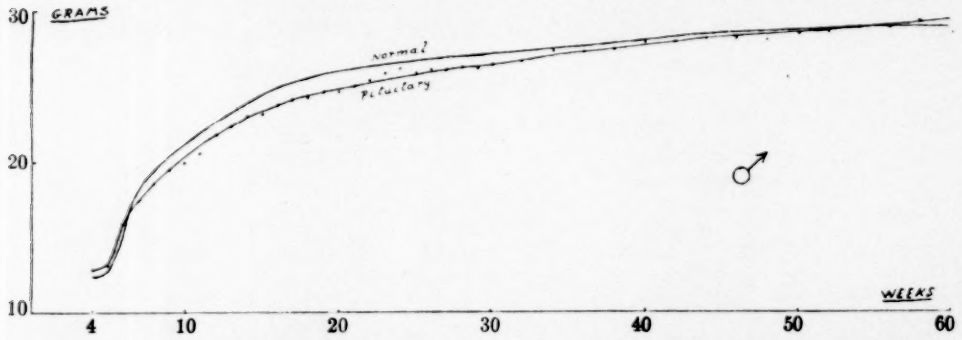

FIG. 1. Comparison of the growth curves of normal and of pituitary-fed males.

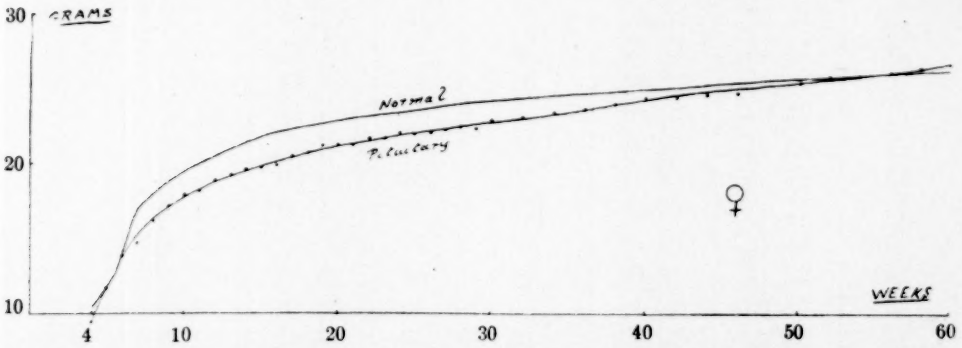

FIG. 2. Comparison of the growth curves of normal and of pituitary-fed females.

up to the normals, but actually, at about 1 year of age, come to surpass the normals in weight.

Summing up the effect of the anterior lobe of the pituitary body upon the rate of growth of mice subsequent to the 4th or 5th week after birth (culmination of the second growth cycle), it may therefore be said that the earlier portion of the third growth cycle is retarded, while the latter portion is accelerated. The action of the pituitary gland is therefore similar in character to

that of other catalysers of growth.[7] Regarding the action of the anterior lobe of the pituitary body upon the first and second growth cycles, these experiments afford no information.

The variabilities of the pituitary-fed and of the normal animals are compared in Tables III and IV and illustrated graphically in Figs. 3 and 4. It will be seen that the effect of the adminis-

TABLE III.

Pituitary-Fed Male Mice.

Age.	Variability.		Age.	Variability.	
	Normal.	Pituitary-fed.		Normal.	Pituitary-fed.
wks.	*per cent*	*per cent*	*wks.*	*per cent*	*per cent*
4	24.4	22.7	25	11.0	9.8
5	24.6	24.1	26	10.8	9.2
6	22.0	22.1	27	11.3	8.8
7	16.9	17.7	28	10.9	8.7
8	15.9	17.3	29	10.9	8.8
9	16.5	14.8	30	9.5	8.4
10	16.7	15.4	32	9.6	9.0
11	13.3	14.9	34	10.5	9.7
12	13.7	11.4	36	10.1	9.3
13	14.2	10.7	38	10.5	9.3
14	14.1	10.0	40	10.4	9.2
15	11.9	9.8	42	11.2	10.6
16	12.7	9.3	44	12.0	10.0
17	12.4	9.9	46	11.7	10.1
18	12.1	9.7	48	10.8	11.1
19	12.1	10.2	50	11.1	11.2
20	10.8	9.8	52	10.7	11.2
21	11.2	9.6	54	12.0	11.4
22	9.0	11.5	56	12.0	11.1
23	9.8	8.7	58	10.9	11.0
24	10.1	10.5	60	11.8	11.0

tration is in both sexes to diminish the variability of the animals, especially between the 10th and the 20th weeks, when the difference in weight of the two groups of animals is also most marked. Between the 20th and 60th weeks, however, the variability curves of both sexes tend to approach the variability curves

[7] Compare the references cited in the early part of the second article of this series.

of the normals, and at about the 40th or 50th week the two variability curves intersect. The effect of the administration upon the variability of the animals is therefore similar to its effect upon the velocity of growth, a phenomenon which finds its explanation in the fact, to which attention is drawn in the preceding article of this series, that the variability of weight increases and decreases

TABLE IV.

Pituitary-Fed Female Mice.

Age.	Variability.		Age.	Variability.	
	Normal.	Pituitary-fed.		Normal.	Pituitary-fed.
wks.	*per cent*	*per cent*	*wks.*	*per cent*	*per cent*
4	23.4	20.5	25	11.4	10.4
5	19.4	19.6	26	11.6	10.5
6	18.9	16.1	27	11.8	10.3
7	15.0	15.8	28	10.3	10.0
8	13.9	15.1	29	12.2	10.3
9	13.4	13.1	30	12.4	9.6
10	14.3	12.1	32	11.5	10.8
11	12.6	10.5	34	12.2	10.8
12	12.6	9.3	36	11.2	10.9
13	13.0	10.3	38	12.3	12.2
14	13.2	9.4	40	11.9	11.8
15	13.7	10.4	42	13.1	12.4
16	12.6	9.3	44	15.3	13.1
17	11.9	9.6	46	14.6	13.2
18	11.9	9.7	48	14.6	13.7
19	12.0	9.7	50	14.0	13.3
20	11.2	9.1	52	15.3	15.4
21	12.3	9.6	54	15.8	15.2
22	12.5	10.2	56	16.5	15.0
23	12.0	9.9	58	16.7	15.9
24	11.3	10.2	60	17.8	14.5

as the velocity of growth increases and decreases. The velocity of growth of the pituitary-fed animals in the earlier portion of the third growth cycle being less than that of the normals, the variability is also less, and as the relative velocity of growth increases so does the variability of weight.

It will be observed that the "period of relative instability" at

the age of about 25 weeks, to which attention was drawn in the preceding article, is also displayed plainly and at the same age in

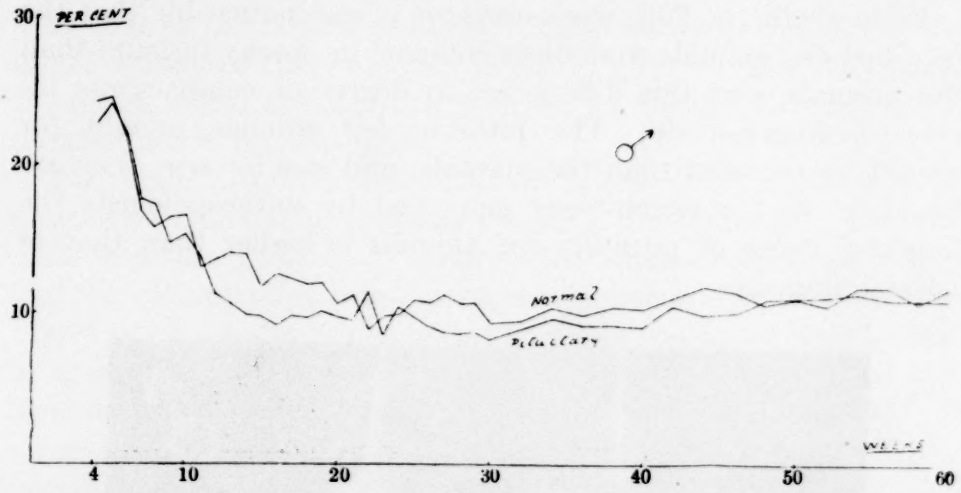

FIG. 3. Comparison of the variability curves of normal and of pituitary-fed males.

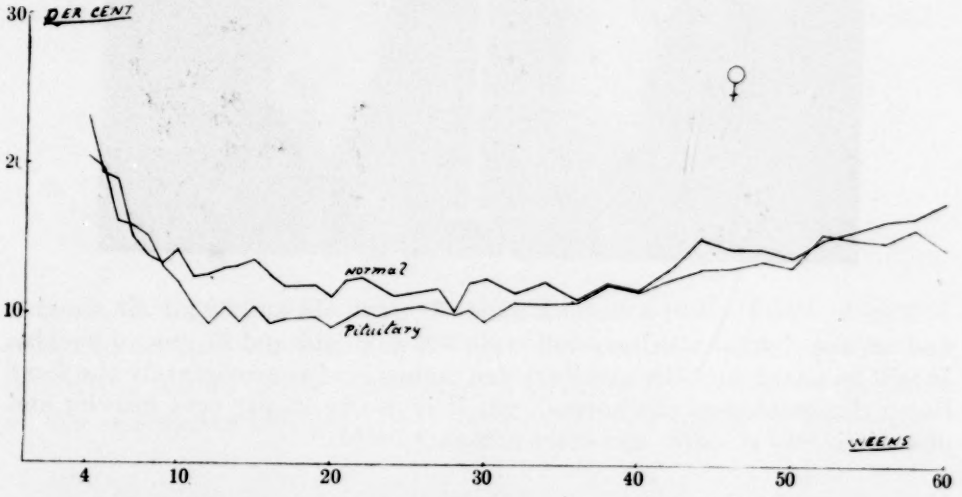

FIG. 4. Comparison of the variability curves of normal and of pituitary-fed females.

the pituitary-fed males by a tendency of the growth curve to oscillate at this point. In the pituitary-fed females this tendency is much less marked.

Differences in the External Appearance and Behavior of the Pituitary-Fed and the Normal Animals.

From about the 30th week onwards it was noticeable that the pituitary-fed animals were more compact or stocky in build than the normals, and this divergence in degree of compactness increased progressively. The pituitary-fed animals, weight for weight, are smaller than the normals, and size for size they are heavier. In the terminology employed by anthropologists the ponderal index of pituitary-fed animals is higher than that of normal animals.

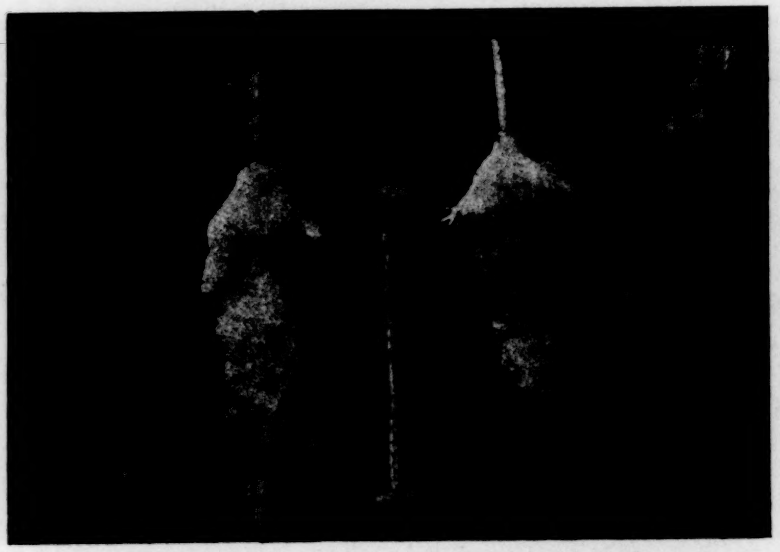

FIG. 5. On the left a normal male 409 days old and 30 gm. in weight, and on the right a pituitary-fed male 396 days old and 37 gm. in weight. It will be noted that the pituitary-fed mouse is of approximately the same linear dimensions as the normal, yet it is nearly 25 per cent heavier and of a distinctly sturdier and more compact build.

This difference is clearly seen in the accompanying photograph (Fig. 5) in which are shown a normal male 409 days old and 30 gm. in weight on the left, and a pituitary-fed male 396 days old and 37 gm. in weight on the right. It will be noted that the pituitary-fed mouse is of approximately the same linear dimensions as the normal, yet it is nearly 25 per cent heavier and is of a distinctly sturdier and more compact build.

The general impression created by the appearance of these animals was that during the earlier growth of the pituitary-fed animals the skeletal framework which was formed was smaller than that of the normal animals.[8] Subsequent to the hardening of the epiphyses these animals began to grow more rapidly than the normals and the accruing weight was, therefore, of necessity packed into a smaller space.

The male pituitary-fed mice also showed much more pronouncedly belligerent propensities than the normals. Normal male mice are sufficiently quarrelsome when confined together, but the pituitary-fed mice surpassed all records in this respect. Many of the males which were otherwise in normal health were in a continually lacerated condition. It was observed that the wounds healed quickly, and that infected wounds were rare.

Analysis of the Causes of Death.

Between the 10th and 60th weeks six deaths were recorded among the pituitary-fed males and three among the pituitary-fed females. The following is a summary of the causes of death:

Males.

Injuries received in fighting	6

Females.

Cancer	2
Unknown causes	1

Comparing the above figures with the two deaths due to injuries received in fighting which were recorded among a similar number of normal males in the same time, a striking quantitative measure of the increased belligerency of pituitary-fed males is obtained.

SUMMARY.

1. The administration of 0.125 gm. per day per animal of fresh anterior lobe pituitary tissue to mice, beginning at 4 weeks after birth (conclusion of the second growth cycle) leads to retardation of growth during the earlier portion of the third growth cycle,

[8] This is confirmatory of previous findings by Cerletti, Sandri, and Etienne and Parisot, referred to above.

between the 6th and the 20th weeks. In the latter part of the third growth cycle, however, from the 20th to the 60th weeks after birth, the growth of the pituitary-fed animals is markedly accelerated, so that they not only catch up to the normals, but actually, at about 1 year of age, come to surpass the normals in weight.

2. The effect of the pituitary tissue upon the variability of the weight of the animals is similar to its effect upon the velocity of their growth. The variability is diminished in the earlier portion of the third growth cycle, particularly between the 10th and the 20th weeks. Between the 20th and the 60th weeks, however, the variability curves of the pituitary-fed animals approach the variability curves of the normals, and at about the 40th or 50th week the two variability curves intersect.

3. Pituitary-fed animals, from about the 30th week onwards, appear more compactly built than normal animals. The pituitary-fed animals, weight for weight, are smaller than the normals of the same age, and size for size they are heavier.

4. Pituitary-fed males are noticeably more belligerent in their habits than normal males.

EXPERIMENTAL STUDIES ON GROWTH.

IV. THE INFLUENCE OF TETHELIN, THE GROWTH-CONTROLLING PRINCIPLE OF THE ANTERIOR LOBE OF THE PITUITARY BODY, UPON THE GROWTH OF THE WHITE MOUSE.

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(Received for publication, January 28, 1916.)

INTRODUCTION.

From the concentrated solution prepared by extracting the dried tissue of the anterior lobe of ox pituitaries with boiling alcohol and evaporating this solution until solid material begins to separate out on cooling, a substance is precipitated by the addition of one and one-half times its volume of dry ether. This substance, after being washed in large volumes of alcohol-ether mixture, may be dried and pulverized and is readily soluble in water. I have called it *tethelin* ($\tau\epsilon\theta\eta\lambda\omega s$, growing) because, as the following experimental data show, its administration to mice is accompanied by effects upon their growth which precisely reproduce the effects, described in the preceding article, which accompany the administration of the whole tissue of the anterior lobe of the pituitary body.

The chemical characteristics, physiological actions other than that upon growth, and details of the method of preparing this substance are fully described in the article which immediately succeeds this. The purpose of the present article is solely to describe the influence which its administration exerts upon the growth of white mice.

The yield of tethelin from 300 anterior lobes was found in a number of instances to lie between 2.6 and 3.0 gm. Taking the higher figure, thus making allowance for possibly incomplete yields, we find the average content of tethelin in each anterior lobe to be 10 mg.

Keeping in mind the possibility that the above estimate might be considerably below the true mark (since tethelin is extracted very slowly from the dried tissue even by boiling alcohol), I thought it well, in initiating these experiments, not to adopt it as a basis of dosage nor to attempt to give to the experimental animals amounts of tethelin equal to those presumably contained in the pituitary tissue supplied to the pituitary-fed animals. It was thought better to err on the side of safety and consequently the amount administered was 50 mg. per 12 mice, corresponding, if the above estimate is nearly correct, as I now believe it to be, to no less than five anterior lobes per day per 12 mice. To the pituitary-fed animals only one anterior lobe was administered per day to 12 mice. Consequently the amount of tethelin received by these animals was nearly five times that which was received by the pituitary-fed animals, a fact which must be carefully borne in mind when the quantitative aspects of the results are under consideration.

Owing to the large demands upon the available material which were made by the chemical investigation of this substance, it was not found possible to spare a sufficient amount of tethelin continually to supply a large number of animals with so large a daily dose.

Two separate experiments were performed. For the one 24 male animals were chosen at random from among our stock, and these received the full dose daily (excepting on Sundays) and without interruption from the age of 5 weeks until the age of 60 weeks. For the second experiment 24 females were similarly chosen. These received the same dosage, but intermittently, in three periods of 1 month's duration each; namely, from the end of the 4th until the end of the 8th, from the end of the 21st until the end of the 25th, and from the end of the 42nd until the end of the 46th weeks.

The tethelin was triturated in a small glass mortar with distilled water in sufficient amount to make a 5 per cent solution. It readily dissolved, forming a cloudy brown so'ution having a characteristic greasy odor reminiscent of that of brain tissue. Not more than 1 week's supply was made up at any one time, since the solution does not keep, and it was stored, when made up, in the ice chest. The mixed whites and yolks of eggs were

beaten and strained, and to 20 cc. of this mixture were added 2 cc. of the tethelin solution. 5 cc. of this mixture were placed in each compartment containing 6 mice. As in all other cases the egg was eagerly eaten by the mice so that all the tethelin administered was consumed.

TABLE I.

Tethelin-Fed Male Mice.

Age.	Weight.		No. weighed. (Tethelin-fed.)	Age.	Weight.		No. weighed. (Tethelin-fed.)
	Normal.	Tethelin-fed.			Normal.	Tethelin-fed.	
wks.	*gm.*	*gm.*		*wks.*	*gm.*	*gm.*	
5	12.45	13.05	20	26	26.94	25 30	23
6	15.58	14.69	24	27	26.55	25.59	23
7	18.08	16.54	24	28	27.19	25.46	23
8	19.36	18.21	24	29	27.08	25.63	23
9	20.63	18.98	24	30	27.23	25.70	23
10	21.19	19.50	24	32	27.61	26.00	23
11	21.81	19.92	24	34	27.71	26.22	23
12	22.65	20.56	24	36	27.95	26.02	23
13	23.31	20.88	24	38	28.20	26.24	23
14	23.96	20.88	24	40	28.27	26.43	23
15	24.28	21.21	24	42	28.28	26.54	23
16	24.75	21.90	24	44	28.62	26.50	23
17	25.21	22.65	24	46	28.55	26.74	23
18	25.61	23.21	24	48	28.22	26.78	23
19	25.81	23.79	24	50	28.88	26.52	23
20	26.10	24.35	24	52	28.68	26.98	23
21	26.28	24.52	24	54	28.67	27.63	23
22	26.06	24.87	23	56	29.32	27.72	23
23	26.34	24.61	23	58	29.10	27.33	23
24	26.82	24.91	23	60	29.08	26.93	23
25	27.05	25.33	23				

In every respect other than in the administration of tethelin these animals were fed and handled exactly as the normal and the pituitary-fed animals were fed and handled. Their cages were kept in the same room as and not far removed from those containing the normal mice. The tethelin-fed males were only slightly (from 2 to 4 weeks) younger than the normals, while the tethelin-fed females were only from 2 to 3 months younger than the normals. Their life and growth were therefore con-

temporary with the greater part of the life and growth of the normal animals. All the animals were invariably weighed at the same time of day (in the middle of the afternoon), so that no possible factor other than the administration of tethelin could account for the observed deviations of these animals from the norm.

TABLE II.

Tethelin-Fed Female Mice.

Age.	Weight.		No. weighed. (Tethelin-fed.)	Age.	Weight.		No. weighed. (Tethelin-fed.)
	Normal.	Tethelin-fed.			Normal.	Tethelin-fed.	
wks.	*gm.*	*gm.*		*wks.*	*gm.*	*gm.*	
4	10.39	10.96	12	25	23.79	21.75	16
5	11.81	13.44	16	26	24.04	22.00	16
6	14.12	14.94	16	27	24.00	21.84	16
7	16.77	15.78	16	28	23.58	22.22	16
8	17.99	16.19	16	29	23.84	22.36	14
9	18.78	16.63	16	30	23.92	22.38	16
10	19.38	17.03	16	32	24.18	22.59	16
11	20.04	17.53	16	34	24.18	23.06	16
12	20.31	17.66	16	36	24.65	22.88	16
13	21.04	18.97	16	38	24.80	23.44	16
14	21.21	19.72	16	40	25.03	23.53	16
15	21.78	20.53	16	42	25.07	23.53	16
16	22.14	20.19	16	44	25.52	23.94	16
17	22.29	20.59	16	46	25.68	23.59	16
18	22.22	20.31	16	48	25.45	23.50	16
19	22.60	20.59	16	50	25.50	24.09	16
20	22.60	20.59	16	52	25.76	24.41	16
21	23.06	21.09	16	54	25.78	24.41	16
22	23.32	21.13	16	56	26.00	24.56	16
23	23.51	21.47	16	58	26.26	24.38	16
24	23.68	21.78	16	60	26.12	24.31	16

Results of the Experiments.

The weights of the tethelin-fed and of normal animals of the same ages are compared in Tables I and II and graphically illustrated in Figs. 1 and 2, the undotted curves representing the normal growth of white mice during the experimental period and the dotted curves the growth of the animals which received tethelin. The dots represent the position of the actual observa-

tions and, in the curve representing the growth of the tethelin-fed females (Fig. 2), the heavily shaded portions of the base line indicate the periods during which tethelin was administered to these animals.

The results, it will be seen, are qualitatively identical with those which accompany the administration of the whole anterior lobe, as described in the preceding communication. From the

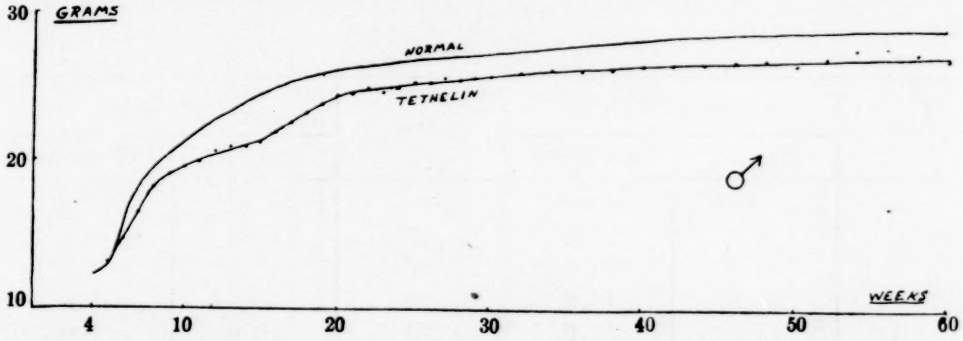

FIG. 1. Comparison of the growth curves of normal and of tethelin-fed males.

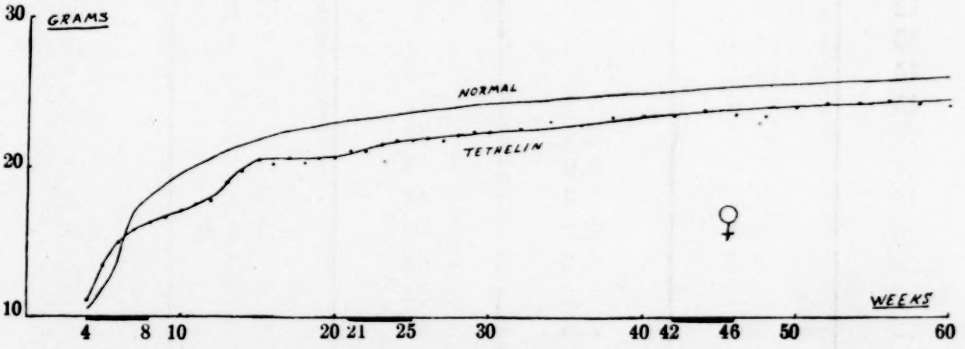

FIG. 2. Comparison of the growth curves of normal and of tethelin-fed females. The heavily shaded portions of the base line indicate the periods during which tethelin was administered to the animals.

5th until the 15th week the growth of the males is greatly retarded. From the 15th week on marked acceleration is evidenced which continues until the 25th week, after which the growth curve of the tethelin-fed animals remains nearly parallel to and somewhat below that of the normals. These effects are so marked as to cause complete distortion of the growth curve, giving the appearance of great prolongation and enlargement of

the second growth cycle and acceleration and curtailment of the third growth cycle.

In females the effects observed are similar, but the retardation is less prolonged, being replaced by acceleration at the 12th week. The acceleration is not so prolonged and tends to disappear by the 20th week, when it is restored by the renewed administration of tethelin from the 21st to the 25th weeks. There-

TABLE III.

Tethelin-Fed Male Mice.

Age.	Variability.		Age.	Variability.	
	Normal.	Tethelin-fed.		Normal.	Tethelin-fed.
wks.	*per cent*	*per cent*	*wks.*	*per cent*	*per cent*
5	24.6	21.8	26	10.8	8.1
6	22.0	18.2	27	11.3	8.0
7	16.9	14.6	28	10.9	7.4
8	15.9	12.0	29	10.9	7.9
9	16.5	10.2	30	9.5	7.2
10	16.7	10.8	32	9.6	7.1
11	13.3	10.9	34	10.5	8.8
12	13.7	10.6	36	10.1	6.9
13	14.2	10.5	38	10.5	7.4
14	14.1	11.2	40	10.4	5.4
15	11.9	9.1	42	11.2	5.8
16	12.7	8.4	44	12.0	6.9
17	12.4	8.0	46	11.7	7.0
18	12.1	7.3	48	10.8	6.9
19	12.1	7.2	50	11.1	7.8
20	10.8	6.3	52	10.7	7.2
21	11.2	6.2	54	12.0	7.0
22	9.0	7.6	56	12.0	7.7
23	9.8	6.4	58	10.9	6.7
24	10.1	7.7	60	11.8	7.3
25	11.0	6.7			

after, with some oscillations, the growth curves of the tethelin-fed and of the normal animals remain nearly parallel.

Summing up the effect of the administration of tethelin upon the rate of growth of mice subsequent to the 4th or 5th week after birth (culmination of the second growth cycle), it may be said that the earlier portion of the third growth cycle is retarded, while the latter portion is accelerated. The differences observed

between the effects exerted by the whole anterior lobe of the pituitary body and those exerted by tethelin are purely quantitative and undoubtedly depend upon the different dosages employed in the two experiments. The milder dosage employed in the pituitary feeding experiments produced an initial retardation of the third growth cycle which was not irremediable, and hence the later acceleration of growth not only restored the weight of

TABLE IV.

Tethelin-Fed Female Mice.

Age.	Variability.		Age.	Variability.	
	Normal.	Tethelin-fed.		Normal.	Tethelin-fed.
wks.	*per cent*	*per cent*	*wks.*	*per cent*	*per cent*
4	23.4	27.6	25	11.4	12.8
5	19.4	19.6	26	11.6	13.5
6	18.9	19.4	27	11.8	13.6
7	15.0	16.1	28	10.3	14.2
8	13.9	12.6	29	12.2	14.1
9	13.4	12.4	30	12.4	15.4
10	14.3	13.2	32	11.5	15.4
11	12.6	15.7	34	12.2	15.7
12	12.6	16.6	36	11.2	15.7
13	13.0	14.1	38	12.3	15.6
14	13.2	11.9	40	11.9	16.9
15	13.7	11.4	42	13.1	17.9
16	12.6	11.2	44	15.3	18.8
17	11.9	11.5	46	14.6	17.8
18	11.9	10.7	48	14.6	17.1
19	12.0	11.9	50	14.0	17.6
20	11.2	12.1	52	15.3	16.6
21	12.3	11.2	54	15.8	17.2
22	12.5	11.1	56	16.5	17.1
23	12.0	12.2	58	16.7	16.7
24	11.3	12.7	60	17.8	16.8

the animals to normal but to a level slightly in excess of normal. The greater dosage employed in the tethelin feeding experiments led to so extreme an initial inhibition of growth that the lost ground could not be wholly made up by the subsequent marked acceleration of growth.

The variabilities of the tethelin-fed and of the normal animals are compared in Tables III and IV and illustrated graphically

in Figs. 3 and 4.[1] It will be seen that the effect of the administration in the males is to diminish greatly their variability. The effect of tethelin upon variability is therefore identical with the

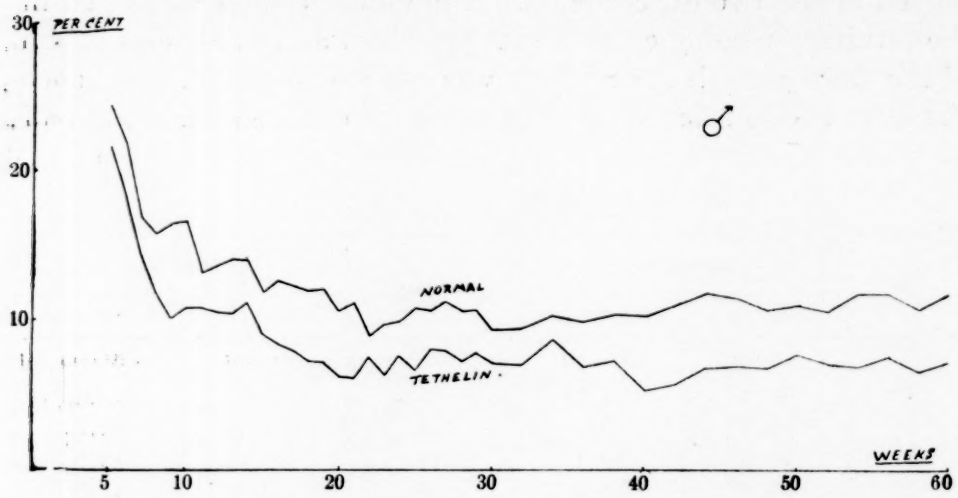

FIG. 3. Comparison of the variability curves of normal and of tethelin-fed males.

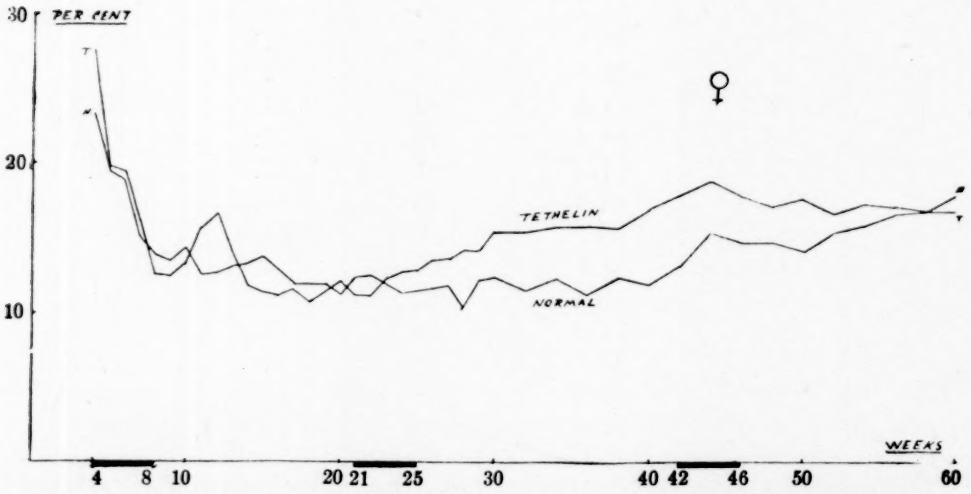

FIG. 4. Comparison of the variability curves of normal and of tethelin-fed females. The heavily shaded portions of the base line indicate the periods during which tethelin was administered to the animals.

[1] In Fig. 4 the variability curve of the tethelin-fed animals is slightly distorted at 20 weeks by the omission of one measurement. This is done to avoid intersection of the two curves at an angle and consequent confusion.

effect of the anterior lobe of the pituitary body upon variability[2] and arises from the same cause; namely, the retardation of the rate of growth. In the females this would appear at first sight not to be the case, for the variability curve of the tethelin-fed females, after lying somewhat below that of the normals from the 8th to the 20th weeks, thereafter lies considerably above the variability curve of the normals. This is, however, due to the fact that the initial variability of the tethelin-fed group of females happened to be much greater than the initial variability of the normal group, and whether we diminish all of the observed variabilities of the tethelin-fed group by the amount of the initial excess, or express all the observed values as percentages of the initial variability, we obtain the same result; namely, that the variability of the tethelin-fed animals was diminished by the administration and strikingly so between the 8th and the 20th weeks.

Differences in the External Appearance and Behavior of the Tethelin-Fed and the Normal Animals.

The differences in build observed and commented upon in the preceding article between the pituitary-fed and normal animals were displayed in even more striking degree by the tethelin-fed animals, and particularly by the males. Their compactness of build is indeed remarkable. Weight for weight they are smaller and size for size much heavier than the normals. The contours of their surface are more rounded and youthful and, most remarkable of all, the coats of the males, even at 14 months of age, retain the glossy, silky appearance of the coats of young animals or of females, while 6 months or more prior to this age the coats of normal males are already shaggy, staring, and discolored.

These differences are clearly displayed in the accompanying photograph (Fig. 5) in which a normal and a tethelin-fed male of the same age (1 year) and of the same weight (28.0 gm.) are compared. The normal animal on the left has a shaggy, staring, and discolored coat, while the tethelin-fed animal has a smooth, glossy, and pure white coat. The normal animal is irregular in

[2] Compare the preceding article of this series.

outline and loosely built, while the contour of the tethelin-fed animal is rounded and its build is compact and stocky.

It may here be mentioned that the improvement in the appearance of the coat which is so striking in the tethelin-fed animals was not observed in the pituitary-fed animals. Whether this difference was a matter of dosage merely, or due to the deleterious action of the meat contained in the dietary of the pituitary-fed animals upon their coats, I cannot say. Another and

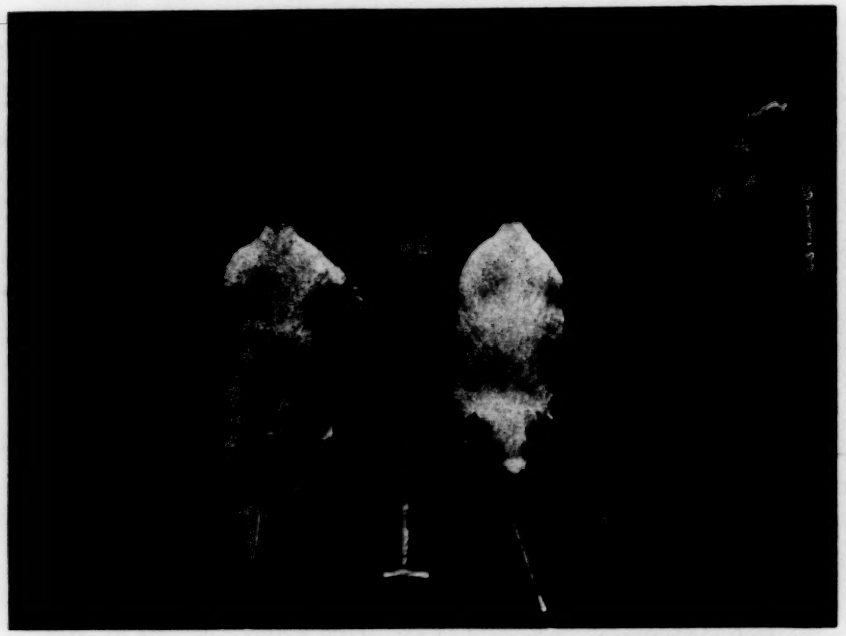

FIG. 5. Comparison of a normal (left) and a tethelin-fed male (right), both 1 year old and 28 gm. in weight. Note the smooth coat and compact form of the tethelin-fed mouse as contrasted with the loose form and rough coat of the normal animal.

striking difference was also noticed between the pituitary-fed and the tethelin-fed animals. The pituitary-fed males, as I pointed out in the preceding article, were remarkably and exceptionally belligerent in their behavior. The tethelin-fed males, on the contrary, were the quietest and least belligerent males in our collection. It would appear that the substance or substances in the tissue of the anterior lobe of the pituitary body which are responsible for the remarkable pugnacity which develops upon its admin-

istration to mice are not identical with or necessarily related to the growth-controlling constituents.

On reviewing these results there can remain little doubt that tethelin is really the growth-controlling constituent of the anterior lobe of the pituitary body. But lest any additional confirmation should be desired I may mention that Dr. Theodore C. Burnett and I have found that hypodermic administrations of tethelin to rats inoculated with carcinoma cause the same remarkable acceleration of the growth of the tumors which we have previously found to be the characteristic effect of similar administrations of the anterior lobe of the pituitary body.[3]

Analysis of the Causes of Death.

Between the 10th and the 60th weeks one death, from injury received in fighting, was recorded among the 24 tethelin-fed males, and one, from paratyphoid, among the 17 tethelin-fed females.

SUMMARY.

1. From the concentrated alcoholic extract of dried anterior lobes of ox pituitaries a substance may be precipitated by the addition of one and one-half volumes of dry ether. This substance I have termed *tethelin*.

2. The effects of tethelin upon the growth of white mice resemble in every particular the effects of the administration of the whole anterior lobe. These effects consist in marked retardation of the first portion of the third growth cycle followed by acceleration of the latter portion of the third growth cycle.

3. The variability in weight of the tethelin-fed animals, like that of the pituitary-fed animals, is less than that of normals of the same age.

4. Adult tethelin-fed animals, like adult animals which have been fed upon the anterior lobe of the pituitary body, are more compact in form and build than normal animals of the same age. Weight for weight the tethelin-fed animals are smaller than the normal ones, and size for size they are heavier.

[3] Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1915, xxi, 280.

5. The coats of adult male animals which have been fed with tethelin retain, even at 14 months of age, the glossy, silky appearance of the coats of young animals. Normal males, even 6 months prior to this age, have shaggy, staring, and discolored coats. This improvement in the coat was not observed in the pituitary-fed animals.

6. Tethelin-fed animals do not display the remarkably enhanced belligerency which is exhibited by pituitary-fed animals.

ON THE ISOLATION AND PROPERTIES OF TETHELIN, THE GROWTH-CONTROLLING PRINCIPLE OF THE ANTERIOR LOBE OF THE PITUITARY BODY.

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PLATE 1

(Received for publication, January 28, 1916.)

Method of Preparation.

Fresh ox pituitaries are stripped of their connective tissue capsules and the anterior lobes are separated from the posterior lobes and from the parts connecting the two lobes. The anterior lobes are then ground up in a mortar with three times their own weight of a mixture of equal parts by weight of anhydrous sodium and calcium sulfates[1] and the mixture is dried over a water bath. It is stirred occasionally to prevent caking. Drying under these conditions is extremely rapid and involves a minimum of exposure of the tissue to heated air. In the course of an hour a mixture of 60 gm. of tissue and 180 gm. of sodium and calcium sulfates becomes white and easily powdered. This dried mixture is returned to the mortar and pulverized as finely as possible and is then extracted with absolute alcohol at a temperature as near to that of boiling alcohol as possible.

For this purpose I employ a modified form (as described on page 379) of the Bailey-Walker extraction apparatus.[2] We obtain the apparatus from Eimer and Amend, New York. One of the extraction thimbles holds the dried powder derived from about six glands. 55 cc. of alcohol are employed in each flask.

In order to obtain a complete yield I find it necessary to carry on the extraction continuously for not less than 48 hours.

The mixed alcoholic extract obtained from 300 glands is filtered

[1] Compare Leathes, J. B., The Fats, London, 1910, 53.

[2] Walker, P. H., and Bailey, L. H., *J. Ind. and Eng. Chem.*, 1914, vi, 497.

to remove the small amount of powder which has fallen into it during extraction, and is then evaporated under reduced pressure until reduced to a volume of about 800 cc., when solid material begins to separate out on cooling. When the concentrated extract is nearly cool it is placed in a tall narrow stoppered glass cylinder, mixed with one and one-half times its volume of dry ether, and the mixture is shaken. A flocculent precipitate appears in the mixture immediately and quickly settles out. After allowing this mixture to stand for several hours the supernatant liquid is siphoned off from the precipitate which is then suspended in somewhat over 2 liters of a mixture of one part by volume of absolute alcohol and one and one-half parts of dry ether and thoroughly shaken. After again allowing the precipitate to settle the supernatant fluid is siphoned off and the precipitate and residual fluid are transferred as quickly as possible to a thoroughly dry hardened filter paper in a thoroughly dry incubator over sulfuric acid, the temperature of the incubator being maintained at about 30–35°C. After a few minutes the incubator is momentarily opened to remove the alcohol-ether which has passed through the filter paper in the interim, and the filter and contained precipitate are allowed to dry for 24 to 48 hours. At the end of this period the dry substance, now in the form of small brittle and readily pulverizable cakes of a pale cream color, is transferred as rapidly as possible to a thoroughly dry glass container.[3]

The substance thus prepared would appear, from the constancy of its nitrogen and phosphorus content, to be a chemical unit. In the communication which immediately precedes this it has been shown that this substance exerts the same influence upon the growth of mice as the whole anterior lobe of the pituitary

[3] Better still, we may employ an apparatus which I have recently had constructed for this purpose, consisting of a large cylinder provided with a clamped lid, capable of withstanding an external pressure of one atmosphere, and provided with automatic electrical temperature control. Sulfuric acid is placed in the bottom of this, the filter funnel and a receptacle for drainage are placed on a false bottom above the acid, the lid is clamped on, and the cylinder is evacuated. The temperature being maintained at a point lying between 30 and 40°C., the ether and alcohol boil off, and when this is completed the vessel is allowed to remain evacuated at the above temperature for 24 hours.

body. It would therefore appear to be the growth-controlling principle of the gland, and for this reason I have termed it *tethelin* ($\tau\epsilon\theta\eta\lambda\omega s$, growing).

Properties.

Tethelin, prepared in the above manner, is a white or pale cream colored substance which is readily powdered. It rapidly absorbs aqueous vapor when exposed to damp air and becomes moist and darkens in color.

When heated it begins to darken at a temperature lying between 100 and 110°C. When heated to still higher temperatures the substance progressively darkens and softens as the temperature rises.

Tethelin is soluble in water to the extent of about 5 per cent, forming at that concentration a brown turbid solution. More dilute solutions are paler in color, and 1 per cent solutions are but slightly opalescent. Aqueous solutions of tethelin have a greasy odor, somewhat like that of brain tissue.

Tethelin is soluble in ethyl alcohol and, to a less extent, in ethyl ether, the solutions in ether being markedly opalescent at high dilutions. It is also soluble in chloroform and in carbon tetrachloride. It is insoluble in a mixture of one part by volume of absolute ethyl alcohol and one and one-half parts of dry ether.

Aqueous solutions of tethelin are faintly acid in reaction; 1 gm. of the substance, dissolved in 200 cc. of water, requiring the addition of about 18×10^{-6} equivalents of sodium or potassium hydroxide (11 mg. of potassium hydroxide) to render the solution neutral to phenolphthalein.

The refractive index of a 1 per cent aqueous solution of tethelin exceeds that of distilled water at the same temperature by 0.00176.

Composition.

The phosphorus content of five separate samples from different preparations of tethelin was determined by von Wendt's modification of Neumann's method.[4] The precipitate of phosphomolybdic acid was dissolved in 0.1 N KOH and titrated to neu-

[4] Von Wendt, G., *Skandin. Arch. Physiol.*, 1905, xvii, 217.

trality to phenolphthalein by the addition of 0.1 N HCl, employing the factor: 1 cc. of 0.1 N KOH = 0.25357 mg. P_2O_5.[5]

The following were the results obtained:

0.230	gm.	substance	yielded	7.568	mg.	P_2O_5,	hence	P = 1.44	per	cent.
0.240	"	"	"	7.600	"	"	"	P = 1.38	"	"
0.195	"	"	"	6.238	"	"	"	P = 1.40	"	"
0.425	"	"	"	13.566	"	"	"	P = 1.39	"	"
0.410	"	"	"	13.312	"	"	"	P = 1.42	"	"
								Mean content, P = 1.41	"	"

The nitrogen content of two samples from different preparations of tethelin was determined by the ordinary Kjeldahl method with the following results:

0.900	gm.	substance	yielded	0.02478	gm.	N,	hence	N = 2.75	per	cent.
0.760	"	"	"	0.02128	"	"	"	N = 2.80	"	"

The nitrogen content in three other samples was determined by the Gunning-Arnold modification of the Kjeldahl method, with the following results:

0.418	gm.	substance	yielded	0.0108	gm.	N,	hence	N = 2.58	per	cent.
0.479	"	"	"	0.0116	"	"	"	N = 2.42	"	"
0.395	"	"	"	0.0094	"	"	"	N = 2.38	"	"
				The mean of the five determinations is				N = 2.58	"	"

These results yield the ratio P : N = 1 : 4.05, or, almost exactly, 1 : 4.

Amino Nitrogen Content.

The amino nitrogen content of three samples of tethelin was determined by the method of Van Slyke,[6] employing for each estimation 10 cc. of a 5 per cent solution. The following were the results:

0.50	gm.	substance	yielded	11.4	cc.	moist nitrogen at 25° and 748 mm. Hence N = 6.22 mg. = 1.24 per cent.				
0.50	"	"	"	11.4	"	moist nitrogen at 25° and 748 mm. Hence N = 6.22 mg. = 1.24 per cent.				
0.50	"	"	"	11.3	"	moist nitrogen at 23.5° and 748 mm. Hence N = 6.22 mg. = 1.24 per cent.				

[5] Neumann, A., *Arch. Anat. u. Physiol., Physiol. Abt.*, 1900, 163.

[6] Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.

The content of amino nitrogen is therefore 1.24 per cent, or almost exactly one-half of the total nitrogen. Since for every phosphorus atom there are four nitrogen atoms present in the substance, it is evident that two of these nitrogen atoms are present in amino groups.

Increase of Amino Nitrogen after Hydrolysis.

The molecule of tethelin also contains one imino group (per atom of phosphorus) which is converted into an amino group by hydrolysis as the following experiments show:

2.5 gm. of tethelin were triturated in 10 cc. of distilled water, 40 cc. of a saturated solution of barium hydroxide were added, and the mixture was boiled under a reflux condenser. At the end of 6 hours a 10 cc. sample, containing the soluble products of the hydrolysis of 0.50 gm. of tethelin, was placed in the Van Slyke apparatus.

Soluble products from 0.50 gm. substance yielded 15.1 cc. moist nitrogen at 23° and 750 mm. Hence N = 8.31 mg. = 1.66 per cent.

It was inferred from this result that hydrolysis was still incomplete. Accordingly, to 30 cc. of the remainder of the solution were added 30 cc. of saturated barium hydroxide solution and the mixture was boiled for 16 hours. At the end of that period two 10 cc. samples were analyzed.

Soluble products from 0.25 gm. substance yielded 8.1 cc. moist nitrogen at 21° and 754 mm. Hence N = 4.55 mg. = 1.82 per cent.
Soluble products from 0.25 gm. substance yielded 8.2 cc. moist nitrogen at 21° and 754 mm. Hence N = 4.61 mg. = 1.84 per cent.

The mean of these estimates is 1.83 per cent, or nearly three-fourths of the total nitrogen content of tethelin.

Summarizing these results we may infer that tethelin contains four atoms of nitrogen for every atom of phosphorus, two of these atoms of nitrogen being contained in amino groups and one in an imino group which is converted into an amino group by hydrolysis with barium hydroxide.

Saponification Value.

A sample consisting of 0.89 gm. of tethelin was mixed with 25 cc. of 0.5 N alcoholic potash solution and heated to boiling under a reflux condenser for 1.5 hours. The substance at the end of that time had not dissolved. 25 cc. of water were added and the substance dissolved almost immediately. Boiling was continued for 1 hour longer, alcohol was then added in sufficient amount to render the final concentration of alcohol at the end of titration about 50 per cent, and the mixture was titrated with 0.5 N HCl, with phenolphthalein as indicator. A blank was treated in exactly the same way with the omission of the tethelin. The result follows:

0.89 gm. substance yielded saponification products neutralizing 85.93 mg. KOH. Hence the saponification value per gm. of substance is 96.55 mg. of KOH.

It was feared that in the above determination hydrolysis might not have been complete. Accordingly another sample, consisting of 0.56 gm. of substance, was dissolved in 50 cc. of 0.5 N aqueous KOH solution. The mixture was boiled for 24 hours under a reflux condenser. At the end of this period a slight incrustation had formed at the edge of the boiling liquid. 25 cc. of water were added and this at once dissolved. The mixture was boiled for 18 hours longer. 50 per cent of alcohol was then added and the mixture titrated as in the preceding experiment. A blank was treated in exactly the same way with the omission of the tethelin. The result follows:

0.56 gm. substance yielded saponification products neutralizing 55.238 mg. KOH. Hence the saponification value per gm. of substance is 98.64 mg. of KOH.

The mean value of these two determinations yields a saponification value of 97.6 mg. of KOH per gm. of substance.

This low saponification value indicates a high molecular weight of the alcoholic radicle or radicles.

From the acid value (11 mg. of KOH per gm.) cited above, we may compute the ester value to be about 87 mg. of KOH per gm. of substance.

Iodine Value.

The iodine value was determined in two samples of material by the method of Wijs.[7] In the first determination the sample was dissolved in 15 cc. of chloroform; in the second the sample was dissolved in 10 cc. of carbon tetrachloride. The following were the results:

0.317 gm. substance absorbed 103.9 mg. iodine; absorption value = 32.8 per cent.
0.257 " " " 85.97 " " " " = 33.5 per cent.

The mean absorption value was therefore 33.2 per cent.

It was observed that on allowing powdered tethelin to stand in contact with air containing traces of moisture for some weeks it became darker in color. The iodine value of a sample of substance which had been thus exposed to air for about 6 weeks was therefore determined with the following result (with 10 cc. of carbon tetrachloride as solvent):

0.295 gm. substance absorbed 64.48 mg. iodine; absorption value = 21.9 per cent. Hence the iodine absorption value of tethelin decreases markedly on standing in contact with air containing traces of moisture.

Reactions.

Tethelin, in aqueous solution, does not yield the biuret reaction. It yields an atypical reaction with Millon's reagent; that is, on the addition of a very small quantity of the reagent to an aqueous solution of tethelin a precipitate appears which turns pink on heating. This color is discharged by adding an excess of the reagent.

When tethelin in aqueous solution, either before or after saponification by barium hydroxide or boiling in barium hydroxide solution followed by boiling in dilute sulfuric acid solution ($\frac{N}{3}$), is boiled with nitric acid and the resulting mixture is evaporated to dryness, a yellow residue is left, which, on exposure to the vapors of ammonia, becomes a deeper yellow.

[7] Leathes, J. B., The Fats, London, 1910, 67.

Aqueous solutions of tethelin do not reduce Fehling's solution either before or after hydrolysis with barium hydroxide or barium hydroxide followed by sulfuric acid.

When tethelin is heated with strong sulfuric acid and sugar, no red or violet color is developed.

Aqueous solutions of tethelin give Ehrlich's reaction;[8] namely, a pink coloration on mixing the solution with an equal volume of a 2 per cent solution of *p*-dimethylaminobenzaldehyde in hydrochloric acid of specific gravity 1.09 and allowing the mixture to stand for a few minutes. This reaction is also given after prolonged boiling of the tethelin with barium hydroxide, but is no longer given after preliminary boiling in barium hydroxide solution followed by boiling in dilute sulfuric acid solution. According to Orgler and Neuberg,[9] this reaction indicates the presence of an acetylated oxy-amino acid radicle.

When chlorine is carefully added to an aqueous solution of tethelin, either before or after hydrolysis with barium hydroxide or barium hydroxide followed by sulfuric acid, a pink coloration appears in the mixture which is destroyed by a very slight excess of chlorine.

Aqueous solutions of tethelin, either before or after hydrolysis with barium hydroxide or with barium hydroxide followed by sulfuric acid, yield Wiedel's reaction as modified by Fischer;[10] namely, a red coloration when the solution is saturated with chlorine, boiled, evaporated to dryness, and the residue exposed to the vapors of ammonia, warmed, and allowed to stand. This reaction probably indicates the presence of an iminazolyl radicle, a possibility which becomes of great significance when viewed in the light of the fact that the physiologically active principles of the posterior lobe of the pituitary body are believed to be iminazolyl derivatives.[11]

[8] Ehrlich, P., *Med. Woch.*, 1901, i, 151. Pröscher, F., *Z. physiol. Chem.*, 1901, xxxi, 520. Müller, F., *Z. Biol.*, 1901, xlii, 561.

[9] Orgler, A., and Neuberg, C., *Z. physiol. Chem.*, 1903, xxxvii, 399.

[10] Fischer, E., *Ber. chem. Ges.*, 1897, xxx, 2226.

[11] Compare Barger, G., and Dale, H. H., *J. Physiol.*, 1910–11, xli, 499. Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1911, xliii, 182. Aldrich, T. B., *J. Am. Chem. Soc.*, 1915, xxxvii, 203.

Decomposition Products.

When several volumes of a saturated aqueous solution of barium hydroxide are added to a concentrated aqueous solution of tethelin, a bulky, flocculent precipitate appears in the mixture. This dissolves on heating, forming a yellow solution and simultaneously a granular precipitate. This precipitate resembles a barium soap of an unsaturated fatty acid, or a mixture of barium soaps, in its appearance, insolubility in water, and ability to blacken osmic acid when warmed.

After prolonged boiling under a reflux condenser and removal of the excess of barium hydroxide by means of carbon dioxide, the above solution is found to contain the following fractions.

A. A fraction which is precipitated, after concentration of the above solution by evaporating on a water bath and filtering off the barium carbonate, by the addition to the solution of several times its volume of absolute alcohol. This substance flocculates and settles quickly. When dry it is white, or slightly tinged with yellow, and absorbs moisture from damp air.[12] It is soluble in water and is precipitated from its aqueous solution by alcohol. It also yields a precipitate with lead acetate. It contains barium and phosphorus and also yields Scherer's test for *inosite; i.e.*, a bright red coloration is developed when the substance is dissolved in nitric acid solution of specific gravity 1.2 and an equal volume of 10 per cent calcium chloride solution, and the same volume of 1 per cent platinic chloride solution are added and the mixture is evaporated to dryness in a porcelain dish and heated.[13] This fraction, when freed by repeated resolution and reprecipitation from the more slowly settling portion of the precipitate does not give Ehrlich's reaction with *p*-dimethylaminobenzaldehyde.

A determination of the barium content of this fraction, purified as far as possible by resolution in water and reprecipitation with

[12] It is probable that two substances are precipitated by alcohol. The one precipitated in larger amount flocculates quickly, is white, non-hygroscopic, and not precipitable by lead acetate. The other, smaller in amount, settles more slowly, is yellow in color, hygroscopic, and precipitable by lead acetate.

[13] Salkowski, E., *Z. physiol. Chem.*, 1910, lxix, 478.

alcohol, washing in alcohol and ether, and drying over sulfuric acid, yielded the following result:

0.1568 gm. substance yielded 62.6 mg. $BaSO_4$. Hence Ba = 23.5 per cent.

A determination of the phosphorus content by von Wendt's modification of Neumann's method yielded the result:

0.0172 gm. substance yielded 2.21 mg. P_2O_5. Hence P = 5.6 per cent.

The ratio of Ba : P found in this fraction was therefore 1 : 1.06; in other words, barium and phosphorus are present in equivalent proportions.

B. This fraction is precipitated from the filtrate after removal of Fraction A by the addition of an equal volume of ether. This substance is soluble in water, does not contain either barium or phosphorus, yields Ehrlich's and Scherer's reactions, and is precipitated from its aqueous solution by the addition of an equal volume of a 25 per cent solution of lead acetate. The dried substance is lemon-yellow in color and very hygroscopic.

C. On evaporating the filtrate, after the separation of Fraction B to dryness, taking up as much of the residue as will dissolve in hot absolute alcohol, and adding to this alcoholic solution an equal volume of ether, a substance is precipitated which when dried is orange-yellow in color and very hygroscopic. This substance is soluble in water, yields Ehrlich's and Scherer's reactions, and is not precipitated from its aqueous solution by the addition of an equal volume of 25 per cent lead acetate solution.

D. The portion of the residue which is insoluble in alcohol, mentioned in the preparation of Fraction C, is grayish white when dried. It is soluble in water, insoluble in alcohol or ether, does not yield Ehrlich's or Scherer's tests, and is not precipitated from its aqueous solution by the addition of an equal volume of 25 per cent lead acetate solution.

The mixed decomposition products of tethelin obtained by prolonged boiling in aqueous barium hydroxide solution followed by prolonged boiling in dilute sulfuric acid solution ($\frac{N}{3}$), after removal of the excess of sulfuric acid by means of barium hydroxide and removal of the excess of barium hydroxide by carbon dioxide, yield, upon the addition of lead acetate solution (25 per cent) a

precipitate which redissolves if too much lead acetate is added. After removal of this precipitate the mixture yields a further precipitate upon the addition of basic lead acetate solution. This latter precipitate, when suspended in water and treated with hydrogen sulfide, yields a substance which resembles *dl*-inosite in the following particulars:

It is soluble in water, tastes sweet, and is precipitated from its aqueous solution in the form of white acicular crystals by the addition of several volumes of ethyl alcohol followed by the addition of a sufficient volume of ethyl ether to render the mixture cloudy. A sample of these crystals, purified by resolution and reprecipitation, washed in alcohol and ether, and dried over sulfuric acid, melted with discoloration (in contact with air) at 219°C. (uncorrected).[14]

The aqueous solution of the crystals, when evaporated to dryness with a small quantity of mercuric nitrate, yields, upon heating the residue, a red color which disappears on cooling and reappears on heating.

When a few of the crystals are dissolved in a drop or two of nitric acid of specific gravity 1.2, an equal volume of a 10 per cent solution of calcium chloride, and the same volume of a 1 per cent solution of platinic chloride are added to the solution, this mixture is evaporated to dryness, and the residue heated, a rose-red color appears which disappears on cooling and reappears with a bluish tinge on reheating.

The presence of both phosphorus and inosite in the growth-controlling principle of the anterior lobe of the pituitary body is of great interest in view of the presence of "phytin" (inosite-hexaphosphoric acid) in the rapidly growing parts of plants[15] and in milk,[16] and its probable importance in connection with the growth of tissues.[16]

Physiological Actions of Tethelin, Other Than Its Action upon Growth.

The characteristic physiological phenomena following administration of the posterior lobe of the pituitary body, or extracts

[14] Maquenne, M., *Ann. chim. et phys.*, 6th series, 1887, xii, 95.

[15] Posternak, S., *Compt. rend. Acad.*, 1903, cxxxvii, 202, 337, 439. Schulze, E., and Winterstein, E., *Z. physiol. Chem.*, 1896, xxii, 91; 1904, xl, 120. Neuberg, C., *Biochem. Z.*, 1908, ix, 551, 557. Anderson, R. J., *J. Biol. Chem.*, 1914, xviii, 441; 1915, xx, 493.

[16] Starkenstein, E., *Biochem. Z.*, 1911, xxx, 56.

thereof, consist in a pronounced rise in blood pressure,[17] slowing and strengthening of the heart beat, stimulation of involuntary muscles,[18] and pronounced diuresis.[19] These effects, as the above cited authors and others have shown, are not brought about by anterior lobe administrations.

The intravenous administration of tethelin does not cause any appreciable rise in blood pressure; on the contrary very little effect consequent upon the administration is to be observed. Very large doses (50 mg. per kilo) dissolved in physiological saline and injected intravenously in rabbits (marginal vein of the ear) lead to a slight and transient fall in blood pressure,[20] followed in a few minutes by a return to normal (Fig. 1) and a slight increase in the amplitude of the heart beat (see the early part of the tracing in Fig. 2 which is a continuation, after 2 minutes' interval, of the tracing in Fig. 1). Repeated administrations of this dose at intervals of a few minutes lead on each occasion to a transient fall of blood pressure, while the amplitude of the heart beat increases considerably (Fig. 2). The fall in blood pressure, even after the third administration, making a total dose of no less than 150 mg. per kilo, is not nearly so great as that produced by the inhalation of a whiff of amyl nitrite (see the end of the tracing in Fig. 2).

That the intravenous administration of tethelin does not cause diuresis is shown by the following experiment.

A male rabbit, 6 pounds in weight, was catheterized. The contents of the bladder measured 3.0 cc. The catheter was allowed to remain in the bladder, and after a lapse of half an hour the urine was collected for a period of 1 hour. The amount passed in this hour was 4.5 cc. 7.5 cc. of a 2.5 per cent solution of tethelin in physiological saline (188 mg.) were

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[17] Oliver, G., and Schäfer, E. A., *J. Physiol.*, 1895, xviii, 277. Howell, W. H., *J. Exp. Med.*, 1898, iii, 215, 245. Schäfer, E. A., and Vincent, S., *J. Physiol.*, 1899, xxv, 87.

[18] Dale, H. H., *J. Physiol.*, 1906, xxxiv, 196. Bell, W. B., *Brit. Med. J.*, 1909, ii, 1609.

[19] Magnus, R., and Schäfer, E. A., *J. Physiol.*, 1901-02, xxvii, p. ix. Schäfer, E. A., and Herring, P. T., *Phil. Tr. Roy. Soc., London*, 1906, cxcix, 1.

[20] Hamburger, W. W., *Am. J. Physiol.*, 1910, xxvi, 178, has previously drawn attention to the existence of an alcohol-soluble depressor substance in the anterior lobe.

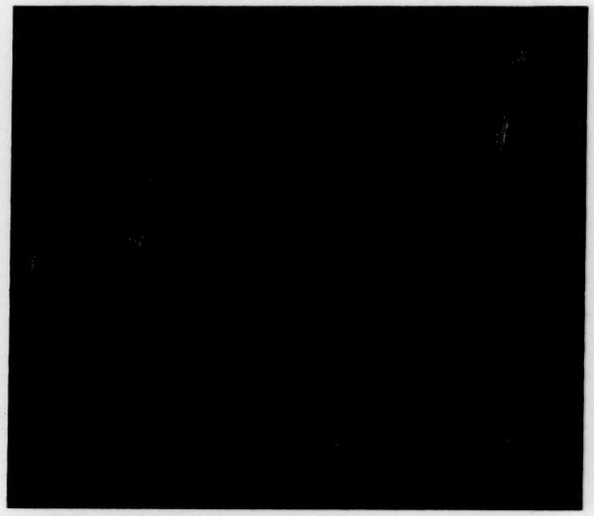

FIG. 1.

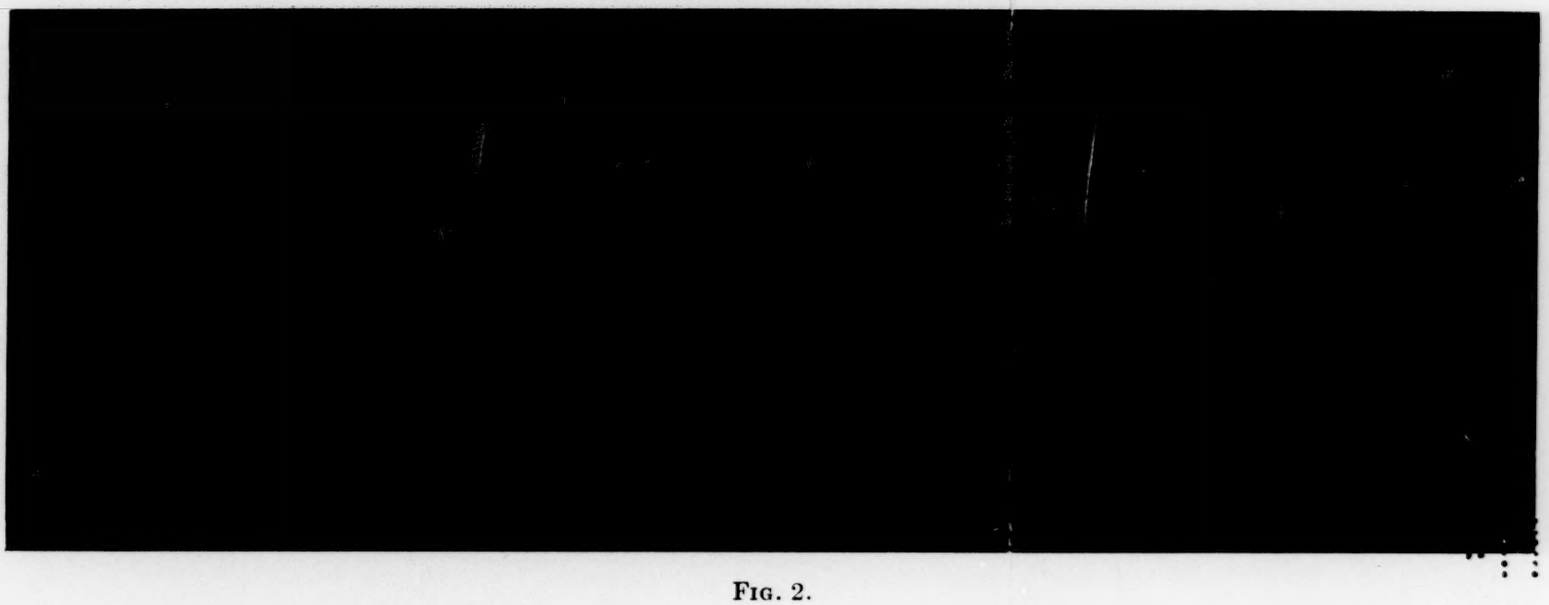

FIG. 2.

FIG. 1. Carotid blood pressure tracing from an ether-anesthetized rabbit, reading from left to right. At the point marked 5 cc., 50 mg. of tethelin per kg. of body weight (125 mg.) dissolved in physiological saline were injected into the marginal vein. The time-marker indicates seconds. Note the transient slight fall in pressure following administration.

FIG. 2. Continuation of the tracing in Fig. 1 after 2 minutes' interval. Note the recovery of normal pressure and slight increase in the amplitude of the heart beat. At the points marked 5 cc., fresh 125 mg. doses of tethelin were administered. Each administration is followed by a fall of pressure which is succeeded in a few minutes by a return to normal pressure and an increase in the amplitude of the heart beat. At the point marked amyl nitrite, a capsule containing 3 minims of amyl nitrite was crushed in a cloth which was held for a few seconds over the animal's nose. The drop in pressure caused by amyl nitrite is much greater than that caused by tethelin.

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now injected into the marginal vein of the ear. During the succeeding hour only 2.0 cc. of urine were passed, and during the hour following that only 1.5 cc. In order to ascertain whether the kidneys of this animal responded normally to diuretic agents, 5 cc. of a 1 per cent solution of barium chloride were now similarly administered. During the hour succeeding this, over 15 cc. of urine were passed.

It is clear, therefore, that at least in so far as blood pressure and diuresis effects are concerned, tethelin does not exert the physiological actions which are characteristic of posterior lobe preparations or extracts.

SUMMARY.

Tethelin, which has been shown in the preceding article to be the growth-controlling principle of the anterior lobe of the pituitary body, is soluble in water, ethyl alcohol, ethyl ether, chloroform, and carbon tetrachloride. It is insoluble in a mixture of one part by volume of absolute alcohol and one and one-half parts of dry ether. It contains 1.4 per cent of phosphorus, and nitrogen in the proportion of four atoms of nitrogen for every atom of phosphorus, two of the atoms of nitrogen being present in amino groups and one in an imino group which is converted into an amino group by hydrolysis with barium hydroxide. Among the products yielded by hydrolysis with barium hydroxide followed by hydrolysis with dilute sulfuric acid is found *dl*-inosite.

Tethelin probably contains an iminazolyl group, and to this extent may be regarded as being related to the physiologically active substances of the posterior lobe of the pituitary body. It does not, however, possess the characteristic physiological activity of these substances, relatively large doses administered intravenously to rabbits (50 mg. per kilo of body weight) producing only a very slight, transient fall in blood pressure and no diuresis.